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Nutritional benefits of microalgae oil replacement in tilapia farming

Submitted by

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SUMMARY

Schizochytrium oil is currently the only commercially available microalgae oil to replace plant and fish oils in Nile tilapia diets, however there are some uncertainties on its benefits and optimal replacement level.

To systematically evaluate the benefits of *Schizochytrium* in fish nutrition, a metanalysis was conducted. Its results indicated that an inclusion of Schizochytrium did not result in loss of omega-3 content of the fish fillet (SMD = 0.62; 95%CI = -0.51-1.76).

To assess the effects on survival and changes in the gut microbiota (16S rRNA sequencing) of Nile tilapia, *Schizochytrium* oil was incorporated in the fish diet. Microalgae-oil in the diet increased growth rate without impacting on fish survival. Plant oil caused an increase in the abundance of *Aeromonadaceae* in the gut, but this was not the case with microalgae oil was used.

To characterize the long-term effects of microalgae oil, Nile tilapia from the previous experiment were fed on the same diets for 11 months. Schizochytrium oil in the diet increased the omega-3 content of the Nile tilapia fillet up to 32.30±1.13%.

The benefits of *Schizochytrium* oil replacement was also tested on Manyara tilapia (*Oreochromis amphimelas*), an endangered species. *Schizochytrium* oil increased fish growth and omega-3 content of the flesh up to 32.68±0.25%. Gonad development was not affected by *Schizochytrium* oil in the diet.

The results of this thesis indicate, that *Schizochytrium* oil can replace fish-based oils in the diet of Nile tilapia without compromising survival or disrupting the composition of the gut-microbiome as plant-based diets do, whileat the same time improving growth and omega-3 content of the fillet in both Nile and Manyara tilapia thus representing a better alternative to fish oils than plant-based oils.

DECLARATIONS AND STATEMENTS

I, **Sergio Trevi**, certify that this work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed 

Date

This thesis is the result of my own investigations except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references, with additional bibliography appended.

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PUBLICATION DECLARATION

The following people and institutions contributed to the publication of work undertaken as part of this thesis

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Chapter 2 Candidate conceived the study, collected the data from the body of literature, carried out the statistical analysis and wrote the first draft of the manuscript.

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MS under review in *Aquaculture Reports*

MS 2: Effects of micro-algae oil replacement on growth, omega-3 deposition

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MS under review in *Aquaculture*

MS3: Long term microalgae oil supplementation increases growth and omega-3 content of Nile tilapia (*Oreochromis niloticus*)

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I am dedicating this thesis to my parents and brother: Francesco, Carla and Federico; I am grateful for their support and to be close to me even if geographically far away. And to my fiancée Sophie for always being with me when I needed her the most.

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DEFINITIONS OF ABBREVIATIONS

A50	Algae 50
A100	Algae 100
ANOVA	Analysis of Variance
bp	Base pair
CF	Condition factor
CI	Confidence interval
cm	Centimetres
CON	Control
Cq	Quantification cycle
df	Degrees of Freedom
FO	Fish oil
GSI	Gonado-Somatic Index
HSI	Hepato-Somatic Index
I^2	Point estimate in meta-analysis
L	Litre
LM	Linear model
m	Meter
m3	Cubic meters
mL	Millilitres
mm	Millimetre
NCBI	National Center for Biotechnology Information
ng	Nanograms
PCR	Polymerase Chain Reaction
PO	Plant oil
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
s	Second
Sc	Schizochytrium
SD	Standard Deviation

SGR	Standard Growth Rate
SMD	Standardised Mean Difference
sp.	Species
Sp	Spirulina
UK	United Kingdom
µl	Microlitres
µg	Microgram
µL	Microlitres
µm	Micrometres
℃	Celsius

CHAPTER 1 - INTRODUCTION

1.1 The need for novel sources of fatty acids in aquaculture

The growth of the aquaculture sector has accelerated since modern fish farming techniques were developed in the 1970's and fish farming will soon overtake capture fisheries as the main source of fish for human consumption, just as livestock farming replaced hunting before (Bostock, McAndrew et al. 2010). With the expansion of the aquaculture sector the logistic and supply chain also expanded as it happened three decades before with the poultry industry (Asche, Cojocaru et al. 2018). The cost of aquafeeds is the limiting factor most aquaculture operations (Naylor, Hardy et al. 2009), followed by water and energy costs (Boyd and Gross 2000).

In the early stages of aquaculture, the reliance on fisheries products like fish meal and fish oil to produce fish feed was very high, however exploitation of wild fish stocks peaked in 1995 and slowly declined thereafter (Shepherd and Jackson 2013). In addition to the needs of the feed industry, human consumption of fish meal and oil has also steadily increased over time due to a global shift towards diets which include more sea-food products, emphasizing the health benefits of fish consumption (Froehlich, Jacobsen et al. 2018). Limited supply and increased demand caused a surge in the costs of fish meal and oil in the past 25 years (Kok, Malcorps et al. 2020), in addition to concerns regarding the environmental sustainability of such pressure on wild fish stocks (Naylor, Hardy et al. 2009).

Acknowledging those issues, the aquaculture-feed industry started a search for alternative feed ingredients in the early 2000's (Rana, Siriwardena et al. 2009).

Fish meal as source of protein for aquaculture was, and still is, replaced mainly by plant protein sources, but their utilization can cause nutritional problems in fish due to poor digestibility, imbalanced profiles of essential fatty acids and the presence of anti-nutritional factors (Jannathulla, Rajaram et al. 2019). Other alternative protein sources which can replace fish meal with various degree success and without the side-effects associated with plant proteins include poultry by-product meal (which is however not allowed by legislation in the EU) (Galkanda-Arachchige, Wilson et al. 2020), *Hermetia illucens* meal also known as black soldier-fly (Magalhães, Sánchez-López et al. 2017, Stadtlander, Stamer et al. 2017), meat meal and blood meal (Millamena 2002) and feather meal (Campos, Matos et al. 2017). Alternatively,

the cyanobacteria species commercially known as Spirulina could be an alternative to fish meal as these microalgae have an high protein content (up to 70%), minerals, vitamins as well as possessing several beneficial characteristics absent in the previously mentioned ingredients like conferring resistance against pathogens and environmental stressors in several fish species (Chamorro-Cevallos and Barrón 2007, Zhang, Man et al. 2020). The current high costs of production remain the only obstacle for this ingredient to become a more commercially viable alternative to fish meal at this time (Rosas, Poersch et al. 2019).

But replacing fish meal is only part of the issue, as an extensive meta-analysis conducted by Cottrell, Blanchard et al. (2020) highlighted that the need to replace fish oil with alternative ingredients is the more pressing than the need to replace fish meal, as more fish biomass is required to produce fish oil than fish meal.

Plant oils from soyabean, linseed, flaxseeds, canola, palm and coconut became the prime candidates to replace marine oils due to their wide availability and use in human and animal nutrition (Turchini, Torstensen et al. 2009). This switch from fish to plant oils is not without its issues, however. Livestock across the globe already rely heavily on plant oils, and the added demand from aquaculture can cause an increase in prices and turn further land into farmland to supply such growing demand (Troell, Naylor et al. 2014). Microalgae, on the other hand, represent some of the most promising and sustainable alternative sources of fatty acids to replace both fish and plant oils (Shah, Lutzu et al. 2018).

1.2 Microalgae in aquaculture nutrition

Microalgae as replacement for marine ingredients possess several advantages compared with plant ingredients: microalgal growth and yields are not affected by seasonality (Park and Lee 2016), they can be grown on industrial wastewater effluents (Singh and Olsen 2011) and do not require freshwater (in the case of marine species) and/or expensive arable land to be produced (Van Krimpen, Bikker et al. 2013). Microalgae have been used as live feed for molluscs, shrimp larvae (Wikfors and Ohno 2001), and some fin-fish larvae (Muller-Feuga, Robert et al. 2003) for many decades but the use of microalgal biomass as a source of oil for fish feed is more recent (Pulz and Gross 2004). The main reason for this late adoption of microalgae as fish feed ingredient is the high cost of industrial production, but recent improvements in technology and know-how are beginning to make it more profitable (Ruiz, Olivieri et al. 2016, El-Dakar, Ramzy et al. 2020). Simply producing algae biomass is often

not enough for aquaculture purposes, as the nutrients of micro algae are contained inside the algal cell-wall which is indigestible by mono-gastric animals like fish (Teuling, Schrama et al. 2017). This stands true for lipids as well which are contained inside the cell wall of the algae, generally making between 1% and 70% of the algal total biomass in weight (Spolaore, Joannis-Cassan et al. 2006). However, increasing interest in microalgae as a potential source of biofuel has prompted the development of effective methods for extracting oil from microalgae, allowing microalgae oils to become available on the market (Wahlen, Willis et al. 2011).

1.3 Lipid content and fatty acid profile of microalgae

Microalgae is an "umbrella term" which includes all monocellular algae invisible to the naked eye, each with its own culture conditions, nutritional properties and fatty acid profile (Muller-Feuga 2000). The fatty acid profile of microalgae tends to be similar within the same *phylum*, but it can vary greatly across different *phyla* (Hu, Sommerfeld et al. 2008). Oil extracted from microalgae generally contains a high percentage of polyunsaturated fatty acids (PUFA) ranging between 20 and 60% (Xue, Yu et al. 2020). The main PUFAs contained in microalgae oil consist of Ω-3 fatty acids, in particular eicosapentaenoic acid (EPA 20:5, n-3) and docosahexaenoic acid (DHA 22:6, n-3) (Ryckebosch, Bruneel et al. 2012).

Several microalgae have a comparable, or even higher, content of PUFAs than most fish species used to produce fish oil (**Table 1.1**). Microalgae are the primary producers of PUFAs, in particular EPA and DHA, as animals are unable to synthetise them in large quantities (Pulz and Gross 2004). In contrast, plant oils can contain a high amount of PUFAs but those consist mostly of alpha-Linolenic acid (ALA), a precursor of EPA and DHA, which has low conversion efficiency in many organisms, including humans (Gómez-Cortés and Camiña 2019). Feeding experiments have demonstrated the beneficial effects of diets rich in DHA and EPA across many vertebrates, from fish to mammals (Zhou, Ding et al. 2018, Luo, Ai et al. 2019). EPA plays an important role in lipid metabolism by preventing diseases like atherosclerosis (Russell and Bürgin-Maunder 2012, Zhang, Wang et al. 2017) as well as being precursors of potent lipid mediators, called eicosanoids, which play an important role in the regulation of the inflammatory responses (Chapkin, Kim et al. 2009).

Table 1.1: Saturated (SFA), monosaturated (MUFA) and polyunsaturated (PUFA) fatty acids content of fish oils compared with microalgae oils

Fatty acids source	SFA (%)	MUFA(%)	PUFA (%)	Reference
Anchovy (*Engraulis encrasicolus*)	31.59±0.12	24.07±0.01	29.57±0.18	Kaya and Turan (2008)
Pacific herring (*Clupea pallasii*)	25.22± 0.17	48.20 ± 0.45	26.57 ± 0.37	Iverson, Frost et al. (2002)
Menhaden (*Brevoortia patronus*)	35.10	24.30	40.60	Lee and Foglia (2001)
Peruvian anchoveta (*Engraulis ringens*)	29.1 ± 1.30	25.8 ± 0.50	45.1 ± 1.20	Standal, Rainuzzo et al. (2012)
Sardine (*Sardinops sagax*)	25.20 ± 4.17	14.21 ± 1.75	56.81 ± 3.64	Huynh and Kitts (2009)
Schizochytrium sp.	22.66 ± 0.76	2.18 ± 0.17	71.64 ± 0.48	Ren, Chen et al. (2018)
Chlorella vulgaris	25.1 ± 0.0	14.8 ± 0.0	60.0 ± 0.1	Canelli, Tarnutzer et al. (2020)
Spirulina maxima	46.31	11.24	40.36	Ötleş and Pire (2001)
Spirulina platensis	51.64	5.88	39.51	Ötleş and Pire (2001)
Tetraselmis sp.	26.43 ± 0.42	36.32 ± 0.83	37.26 ± 0.63	Custódio et al. (2014)
Scenedesmus sp.	25.19 ± 1.09	36.32 ± 0.83	54.94 ± 1.95	Custódio, Soares et al. (2014)
Nannochloropsis sp.	38.8 ± 4.20	32.7 ± 3.00	53.9 ±6.00	Hulatt, Wijffels et al. (2017)

DHA is one of the main constituents of the cell membrane of the neural tissues such as the retina and the brain and thus essential for the development and function of those organs (Politi, Rotstein et al. 2001, Lauritzen, Brambilla et al. 2016).

A high content of DHA in the diet has been associated with downregulation of the inflammatory responses (Alfaddagh, Elajami et al. 2018). When needed, DHA is liberated from the neural cell membranes via specific phospholipases so it can undergo further processing (both enzymatic and non) and being turned into signalling molecules called eicosanoid (Tassoni, Kaur et al. 2008). Particularly important eicosanoids are leukotrienes and prostaglandins, originally discovered in mammals but present also in lower vertebrates and some invertebrates (Lall 2000, Di Costanzo, Di Dato et al. 2019). Binding through specific G protein–coupled receptors (Funk 2001), those eicosanoid act as mediators of leukocyte accumulation during acute inflammation (Yoshikai 2001). In addition to their role as mediators in the inflammatory response, omega-3 fatty acids are also involved in the regulation of various other metabolic pathways (Hussein, Attia et al. 2019) and enhancement of cognitive abilities (Sinn, Milte et al. 2012, Zhou, Ding et al. 2018, Liu, Wang et al. 2020). Omega-3 fatty acids are also essential during gonad development of fish and their presence in diets affect the quality of sperm and egg of teleost fish (Köprücü and Özcan 2019). Study on male Iranian Sturgeon (*Acipenser persicus*) indicates that a diet rich in omega-3 diet maintain in optimal condition spermatological parameters and reproduction processes (Mehdinejad, Taghizadeh et al. 2013) meanwhile omega-3 deficient diets caused incorrect egg development and lower sperm motility in rainbow trout (*Oncorhynchus mykiss*) (Vassallo-Agius, Watanabe et al. 2001). Hybrid catfish (*Pangasius larnaudii* x *Pangasianodon hypophthalmus*), a freshwater species, broodstock displayed higher histological stages of maturity when provided with additional omega-3 in the diet (Sattang, Amornlerdpison et al. 2021) and similar results were found in tongue sole (*Cynoglossus semilaevis*) which is a marine species (Xu, Cao et al. 2017) indicating that the beneficial role of omega-3 (DHA and EPA) in fish gonad development can transferred across species living in different habitats and with different feeding habits.

As EPA and DHA can be transferred through the food chain all the way to the final human consumers (Sissener 2018), microalgae oil can be used to fortify the diets of farmed animals and their valuable polyunsaturated fatty acids can help increase their nutritional value (Ryckebosch, Bruneel et al. 2014).

1.4 Schizochytrium as source of polyunsaturated fatty acids for Aquaculture

Among the microalgae currently being cultivated, the marine and heterotrophic genus *Schizochytrium* is the only microalgae oil which is commercially available for incorporation into fish diets (Tocher, Betancor et al. 2019). *Schizochytrium* possesses two important qualities that make it useful for the aquaculture industry. Its oil has a high content of omega-3, up to 40-45% DHA and 10% EPA (Fedorova-Dahms, Marone et al. 2011). Additionally, and contrary to other microalgae which require very specific carbon sources, *Schizochytrium* can thrive on by-products from agriculture like sugar cane molasses (Carr 2017) or even wastewater from marine aquaculture operations (Jung and Lovitt 2010) making its culture feasible and sustainable. Over the past two decades, *Schizochytrium* has been used in feeding experiments to enhance the growth and omega-3 content of several aquaculture species, including giant grouper (*Epinephelus lanceolatus*) (García-Ortega, Kissinger et al. 2016), seabream (*Sparus aurata*) (Ganuza, Benítez-Santana et al. 2008, Eryalçin and Yildiz 2015), channel catfish (*Ictalurus punctatus*) (Li, Robinson et al. 2009), longfin yellowtail (*Seriola rivoliana*) (Kissinger, García-Ortega et al. 2016), red drum (*Sciaenops ocellatus*) (Kissinger, García-Ortega et al. 2016), hybrid striped bass (*Morone crhysops*♀×*M. saxatilis* ♂) (Perez-Velazquez, Gatlin III et al. 2019), Atlantic salmon (*Salmo salar*) (Miller, Nichols et al. 2007, Kousoulaki, Østbye et al. 2015, Sprague, Walton et al. 2015), Siberia sturgeon (*Acipenser baerii*) (Luo, Ai et al. 2019), jade perch, (*Scortum barcoo*) (Van Hoestenberghe, Fransman et al. 2016), Nile tilapia (*Oreochromis niloticus*) (Sarker, Kapuscinski et al. 2016, dos Santos, Schorer et al. 2019) and red seabream (*Pagrus major*) (Seong, Matsutani et al. 2019, Seong, Kitagima et al. 2020). For marine species, finding alternative sources of PUFAs like *Schizochytrium* is motivated by their limited ability to synthesise long chain poly-unsaturated fatty acids when compared to freshwater fish (Monroig, Webb et al. 2011). However, the ability of *Schizochytrium* enriched diets to enhance the omega-3 content of the fish fillet could also benefit consumers of freshwater fish which generally possess a lower PUFA content (Suito, Nagao et al. 2018). In particular, the nutritional value of freshwater fish that are mostly fed with plant ingredients like Nile tilapia (*Oreochromis niloticus*) could benefit the most (Webster and Lim 2006, Furuya and Furuya 2010). In addition to improving the nutritional value, incorporation of omega-3 rich *Schizochytrium* can also improve development, growth, and reproduction, especially when brood-stock and larvae are involved (Miles and Chapman 2006). Like for other microalgae, the main limiting factor to the adoption of *Schizochytrium* as a

source of polyunsaturated fatty acids for aquaculture in industrial scale operations remains the higher cost compared with other traditional ingredients (Oostlander, van Houcke et al. 2020). One way to reduce the costs of using microalgae in aquaculture is to reduce the amount of time that fish will be fed on *Schizochytrium* feeds while still improving fish growth performances and fillet quality, this can be achieved by feeding the fish the more expensive microalgae diet only for a brief period prior slaughter just like it is already done in the pig and poultry industry were those "finisher diets" are commonplace (Teymouri, Zarghi et al. 2018, Liu, Yan et al. 2019). In a recent study performed by Cortegano, de Alcântara et al. (2019) involving tambaqui (*Colossoma macropomum*), the use of a finisher-diet containing *Schizochytrium* for 71 days before slaughter improved the DHA content and n-3:n-6 ratio in the flesh. Similar results were obtained in Atlantic salmon after 9 or 12 weeks (Miller, Nichols et al. 2007, Kousoulaki, Mørkøre et al. 2016), in sea bream (*Sparidentex hasta*) after two months (Hossain, Al-Adul-Elah et al. 2022), in *Totoaba macdonaldi* after just 6 weeks (Maldonado-Othón, Perez-Velazquez et al. 2020) and in giant grouper (Epinephelus lanceolatus) after 12 weeks (García-Ortega, Kissinger et al. 2016). All those promising results indicate how the use of *Schizochytrium* as ingredient for finisher diets in aquaculture can be a cost-effective way to introduce microalgae-feeds commercially.

1.5 Schizochytrium in Nile tilapia nutrition: current situation, trends, and knowledge gaps

I focussed on Nile tilapia as a study species in this thesis due to its global relevance to the aquaculture sector. Nile tilapia is a freshwater fish native to Africa that was introduced to Asia and the Americas for aquaculture purposes, becoming the second most important farmed fish on the planet (Zambrano, Martínez-Meyer et al. 2006, Bezault, Balaresque et al. 2011, Gu, Ma et al. 2015, Prabu, Rajagopalsamy et al. 2019). The reasons of this success lie on its fast growth, hardiness to adverse conditions, and most importantly, a mostly herbivorous diet which makes tilapia-feed cheap (Köprücü and Özdemir 2005, da Silva Dias, Pereira et al. 2020). However, the high amount of plant-based ingredients in tilapia's diet, especially plant oils, makes the fillets relatively low in Ω-3 fatty acid (Osibona, Kusemiju et al. 2009). This is a serious issue, especially because tilapia is highly consumed in developing countries where nutrient deficiencies can causes problems for children development and well as adult health (Gilani and Nasim 2007). Enriching Nile tilapia feed with Ω3-rich microalgae oil could compensate for the low amount of fish oil in tilapia feeds, and for this reason interest in the potential use of *Schizochytrium* has grown in recent years. Sarker, Gamble et al. (2016) and dos Santos, Schorer et al. (2019) found that the PUFA content of Nile tilapia can be enhanced by supplementation with *Schizochytrium* in the diet, but there is no systematic review of effect sizes and considerable uncertainty on the practical use of *Schizochytrium* as an alternative to conventional fish and/or plant oils in the diet of tilapia. There are also knowledge gaps related to how much conventional oils can be replaced by *Schizochytrium*, what effects this replacement has on the development of the gut microbiome, as well as the effects of microalgae oil on omega-3 content of the fish fillet.

1.6 Potential benefits of *Schizochytrium* oil replacement for other tilapia species

Manyara tilapia (*Oreochromis amphimelas*) is an endangered tilapia which is has been heavily fished and is currently present in only three lakes in Africa (Shechonge, Ngatunga et al. 2019). This general introduction explored whether *Schizochytrium* oil can be used to feed Manyara tilapia and help establish a breeding population. In addition to its potential use in conservation through restocking, microalgae-fed Manyara tilapia could represent a candidate for sustainable commercial aquaculture as it requires less space and matures faster than Nile tilapia. This would lessen pressure on the wild Manyara stocks which are already under threat (Nonga, Mdegela, Lie, Sandvik, & Skaare, 2010). If *Schizochytrium* can increase the omega-3 content of Manyara tilapia's fillets like it does with Nile tilapia, then Manyara tilapia could become an new native source of omega-3 for the local population. This is could be very significant as Tanzania is currently looking for omega-3 sources to combat malnutrition (Charles 2020).

1.7 Novel feed ingredients and fish gut microbiome: balancing a delicate and vital ecosystem

When introducing a new ingredient like *Schizochytrium* in the diet of an organism, consideration must be given to the potential effects on its gut microbial community. This community of organisms, which includes mainly bacteria but also viruses and fungi, is often refereed as gut microbiome and it possess a key role in the health and growth of the organism in which it is hosted (Kinross, Darzi et al. 2011). A well-diversified where every ecological niche is occupied by symbiotic microorganisms, represent an obstacle for the pathogenic ones which are outcompeted and can't establish themselves in numbers high enough to cause damage the host (Weiss 2013). At the same time, many symbiotic species present in the microbiome produces essential vitamins and enzymes which promote the host health and allows it to make the most of its diet (Pan and Yu 2014). However, this equilibrium is delicate, and several stressors can disrupt it, causing losses in its diversity which reduce the production of beneficial metabolic by-products and can promote the growth of potentially pathogenic microbial species within the gut (Moloney, Desbonnet et al. 2014). The factor which by far influences the most the fish gut microbiome is the host diet; Gut microbes depend, either directly or indirectly, on the nutrients ingested by the fish which can promote certain microbial taxa which in turn can outcompete and replace other taxa (Read and Holmes 2017). Any time

the diet of a fish changes, so does its gut microbiome so it's important that the feed ingredients do cause a competitive advantage to potentially pathogenic bacterial species (Llewellyn, Boutin et al. 2014). The study of the fish gut microbiome is a relatively new field, but the microbiome of several farmed fish species has been sequenced like Atlantic salmon (Uren Webster, Consuegra et al. 2018), Nile tilapia (Bereded, Curto et al. 2020), European seabass (Roquigny, Mougin et al. 2021), Rainbow trout (Rimoldi, Terova et al. 2018) and carps (Eichmiller, Hamilton et al. 2016). Several studies in the field of aquaculture-nutrition have also highlighted how changes in the diet can have a harmful effect on the fish gut microbiome, especially when conventional marine ingredients were replaced with plant ingredients (Desai, Links et al. 2012, Parata, Mazumder et al. 2020). The effects on the fish gut microbiome of replacing conventional ingredients with *Schizochytrium* are however not very well known, but some studies in Atlantic salmon (Tibbetts, Scaife et al. 2020) and Nile tilapia (de Souza, de Lima et al. 2020) seem to point towards the conclusion that this microalga does not cause alterations to the normal gut microbiome composition and abundance. If *Schizochytrium* is to become a staple ingredient for the aquaculture industry, then its effects on the gut microbiome of fish need to be further explored and quantified.

1.8Aim and objectives

The aim of this thesis was to investigate the effects of *Schizochytrium* oil replacement on the survival, growth, omega-3 deposition and gut microbiota of Nile tilapia (*Oreochromis niloticus*) and to assess the potential benefits for the culture of Manyara tilapia (*Oreochromis amphimelas*) for conservation purposes and commercial production

Chapter aims and overview

Chapter 2

The aim of Chapter 2 was to review the optimal level of replacement, potential negative effects, and the extent to which the benefits of using the most promising micro-algal alternatives to fish oil (*Schizochytrium*) and to fish meal (*Spirulina*) can be generalised across different fish species by performing a metanalysis of the peer-reviewed literature

S. Trevi, T. Uren Webster, S. Consuegra, C. Garcia de Leaniz. The benefits of microalgae replacement in fish nutrition: a meta-analysis. Under review *in Aquaculture Nutrition*

Chapter 3

The aim of Chapter 3 was to assess the nutritional benefits of of microalgae oil replacement on Nile tilapia fry since first feeding. The effects of *Schizochytrium* oil replacement on the gut microbiome of Nile tilapia was examined through 16s RNA sequencing and the effects on growth, omega-3 deposition and survival were assessed.

S. Trevi, T. Uren Webster, S. Consuegra, C. Garcia de Leaniz. Effects of micro-algae oil supplementation on the growth and microbiota of Nile tilapia (*Oreochromis niloticus*). Under review *in Aquaculture*

Chapter 4

The aim of Chapter 4 was to characterize, for the first time, the long term (11 months) effects of *Schizochytrium* oil replacement on the growth, omega-3 content and expression in the liver of genes related to fish health and lipid metabolism in Nile tilapia.

S. Trevi, T.M. Uren Webster, S. Consuegra, C. Garcia de Leaniz. Long term micro-algae oil supplementation increases growth and omega-3 content of Nile tilapia (*Oreochromis niloticus*). In prep.

Chapter 5

The aim of Chapter 5 was to assess the growth, omega-3 content of the fillet, and female gonad maturation of Manyara tilapia (*Oreochromis amphimelas*) fed on microalgae oil-based diets previously assessed on its close relative, the Nile tilapia.

S. Trevi, T.M. Uren Webster, S.Consuegra, C. Garcia de Leaniz. Effects of micro-algae Schizochytrium oil on the growth performances and omega-3 content of endangered Manyara tilapia (*Oreochromis amphimelas*). In prep.

CHAPTER 2 – THE BENEFITS OF MICROALGAE REPLACEMENT IN FISH NUTRITION: A META-ANALYSIS

2.1 Abstract

Use of microalgae in fish nutrition can relieve pressure on wild fish stocks as a source of fish meal and oil, but there is no systematic quantitative evaluation of microalgae benefits. We conducted a metanalysis on the benefits of two of the most common microalgae used in fish nutrition, *Spirulina* sp. and *Schizochytrium* sp. We reviewed 50 peer-reviewed studies involving 26 different finfish species and compiled effect sizes for 144 control vs micro-algae replacement comparisons in the form of Standardized Mean Differences adjusted for small sample sizes (SMD). Our global analysis under a random model. indicates that inclusion of *Spirulina* in the fish diet can significantly improve growth compared to controls (SMD = 1.21; 95%CI =0.71-1.70) while inclusion of *Schizochytrium* did not result in loss of omega-3 content of the fish fillet (SMD = 0.62; 95%CI = -0.51-1.76). Depending on fish species, benefits were apparent at replacement levels as low as 0.025% in the case of *Spirulina* and 10% in the case of *Schizochytrium* oil. Dose effects were found for *Spirulina* replacement on growth, but not for *Schizochytrium* on omega-3 fillet content. Substantial heterogeneity (I^2 = 89-96%) was found between studies. Subgroup analysis and meta-regression revealed that ~24-27% of variation in effect sizes could be accounted by variation between fish families, but the rest could not be explained by microalgae inclusion levels, differences in fish size, habitat, feeding guild or the way the data were recorded, likely reflecting variation in experimental conditions. Overall, the evidence indicates that *Spirulina* and *Schizochytrium* replacement in aquafeeds can be used to improve fish growth and maintain fillet quality, respectively, but considerable uncertainty exists on the predicted responses. To reduce uncertainty and facilitate the transition towards more sustainable aquafeeds, we recommend that feeding trials using microalgae are conducted under commercially relevant conditions and that greater care is taken to report full results to account for sources of heterogeneity.

2.2 Introduction

Aquafeeds represent the main cost in fish farming, and are also the area where sustainability can improve the most (Naylor, Hardy et al. 2009). The main source of protein and lipids in aquafeeds has traditionally been small marine pelagic fish, as they provide a good balance of the essential amino acids and the omega-3 fatty acids needed by virtually every commercially farmed fish (Miles and Chapman 2006), and the high quality fish fillets needed for human

consumption (Tacon 2004). However, groundfish and small pelagic fish have declined worldwide as a consequence of the demands made by aquaculture industry being too high (Froehlich, Jacobsen et al. 2018).

In response to a shortage of wild fish, the aquafeed industry turned to plant-based ingredients due to their wider availability, lower costs and established knowledge from their use in human and livestock nutrition (Turchini, Torstensen et al. 2009, Bhosale, Bhilave et al. 2010). Plant oils from soyabean, linseed, flaxseeds, canola, palm and coconut became the prime candidates to replace marine oils, but their use in aquafeeds has several nutritional limitations as well as their own sustainability issues (Turchini, Ng et al. 2010). Livestock across the globe already rely heavily on plant oils, and there are fears that further demand from aquaculture could increase prices and turn even more land into farmland (Troell, Naylor et al. 2014). Proteins derived from plants typically lack some of the essential amino acids, like methionine, lysine, and tryptophan present in fish meal, and some contain anti-nutritional factors (Daniel 2018). Anti-nutritional factors include protease inhibitors, phytates, glucosinolates, saponins tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters (Francis, Makkar et al. 2001) and are particularly harmful for carnivorous fish (like Atlantic salmon) who did not evolve to metabolize with them (Oliva-Teles, Enes et al. 2015). At the same time, plant oils are typically deficient in omega-3 fatty acids (Tocher 2015). These limitations are particularly problematic for marine farmed fish, as they cannot synthesize omega-3 fatty acids efficiently and must rely on the diet to obtain them (Zhang, Ning et al. 2020).

In a quest to find more sustainable alternatives to fish products, and more suitable sources of omega-3 fatty acids than plant oils, photosynthetic microalgae and cyanobacteria have received increasing attention (Teuling, Schrama et al. 2019). The protein content and fatty acid composition of some microalgae are similar to those provided by marine pelagic fish, and are superior to those derived from terrestrial plants (Lum, Kim et al. 2013). Recent developments in algal biotechnology have also made the production of microalgae cheaper and more readily available (Koyande, Chew et al. 2019), but there are still challenges concerning upscaling, and knowledge gaps that have prevented their wider use. Early research on microalgae as aquafeeds focused on their use as feed additives, mostly as live cells, but there is increasing interest in their potential value as full or partial replacements of fish oil (Shah, Lutzu et al. 2018) or protein (Ragaza, Hossain et al. 2020).

One microalgae in particular, the genus *Arthrospira* (*Spirulina*), has received much attention as it has a protein content similar to that of marine fish, between 50% and 70% of its dry weight (Ogunji, Kloas et al. 2006, Roy and Pal 2015), as well as a high digestibility due to the lack of a cellulose cell wall (Cao, Zhang et al. 2018, Cao, Zou et al. 2018). With a global annual production of 3,000 Tn dry weight, the *Spirulina* market was worth $394 million in 2019, and is growing at a rate of ~10% annually. It is one of the most intensively farmed microalgae in aquaculture and the species that offers some of the best options for fish protein replacement (Spolaore, Joannis-Cassan et al. 2006). However, *Spirulina* cannot be used as replacement for fish oil, as this requires microalgae with different nutritional profiles. The genus *Schizochytrium* is rich in omega 3 fatty acids (Sarker, Gamble et al. 2016, Sarker, Kapuscinski et al. 2016) and is already produced on an industrial scale as a food supplement (Nguyen, Su et al. 2018). Its use can improve the omega 3 content of fish fillets (Shah, Lutzu et al. 2018) and can be produced in the large quantities required by the salmon farming industry (Tocher, Betancor et al. 2019).

A combination of *Spirulina* and *Schizochytrium* could be used as a replacement of fish protein and fish oil in aquafeeds (Sarker, Gamble et al. 2016), but there is little guidance on optimal levels of replacement, and uncertainty regarding the extent to which the benefits of using microalgae can be generalised across different fish species. Production costs of live microalgae for aquafeeds currently range between 300 and 600 €/kg, and although these could be reduced by 60-80% with upscaling (Oostlander, van Houcke et al. 2020), they are still more expensive than animal feeds(Shah, Lutzu et al. 2018). Crucially, there is no information regarding variation in effect sizes (i.e. variation in the magnitude of any purported microalgae nutritional benefits) and therefore it is not possible to asses to what extent the high costs of producing microalgae can be compensated by improved growth or enhanced fillet quality.

In order to address these questions, we carried a systematic review followed by a metanalysis on the effects of using *Spirulina* and *Schizochytrium* as replacement of fish protein and fish oil in fish feeds. Our paper aimed to address three main knowledge gaps: (1) to assess the extent of variation in the nutritional benefits of two of the main microalgae used in aquafeeds, (2) to gain insights into sources of heterogeneity and (3) to assess the existence of publication bias, as it is important to know if bias against negative results may have exaggerated the nutritional benefits of microalgae-enriched diets.

2.3 Methods

2.2.1 Selection criteria for the systematic review

We adopted the PRISMA protocol (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) as described by Moher, Liberati et al. (2010) for the systematic literature review (**Figure 2.1**). We searched Google Scholar with the keywords "*Spirulina*" AND "SGR" AND "fish" AND "aquaculture" AND "Arthrospira" for the *Spirulina* analysis. This search string returned 627 results. For the *Schizochytrium* analysis, we searched for the keywords "*Schizochytrium"* AND "omega-3" AND "fish" AND "aquaculture", obtaining 1,150 results. The searches were carried out on 08/11/2019 and the timeline was set between the years 2000 and 2020 (inclusive), as before 2000 microalgae were used mainly as whole feed rather than as replacements in aquafeeds.

We used three criteria for selecting articles for subsequent analysis: (1) primary peer-reviewed research papers (i.e. we excluded reviews) carried out on finfish and written in English, (2) studies in which microalgae were used as partial replacement in fish feeds, and not as sole nutrients, and (3) studies that reported the Standard Growth Rate (SGR) for *Spirulina*, or the omega-3 content in the fish fillet for *Schizochytrium,* along with standard errors (or standard deviations) and sample size. We found 36 studies on *Spirulina* (representing k = 101 control-treatment comparisons) and 14 studies on *Schizochytrium* (representing k = 43 control-treatment comparisons) that met these criteria published during the period 2000-2019.

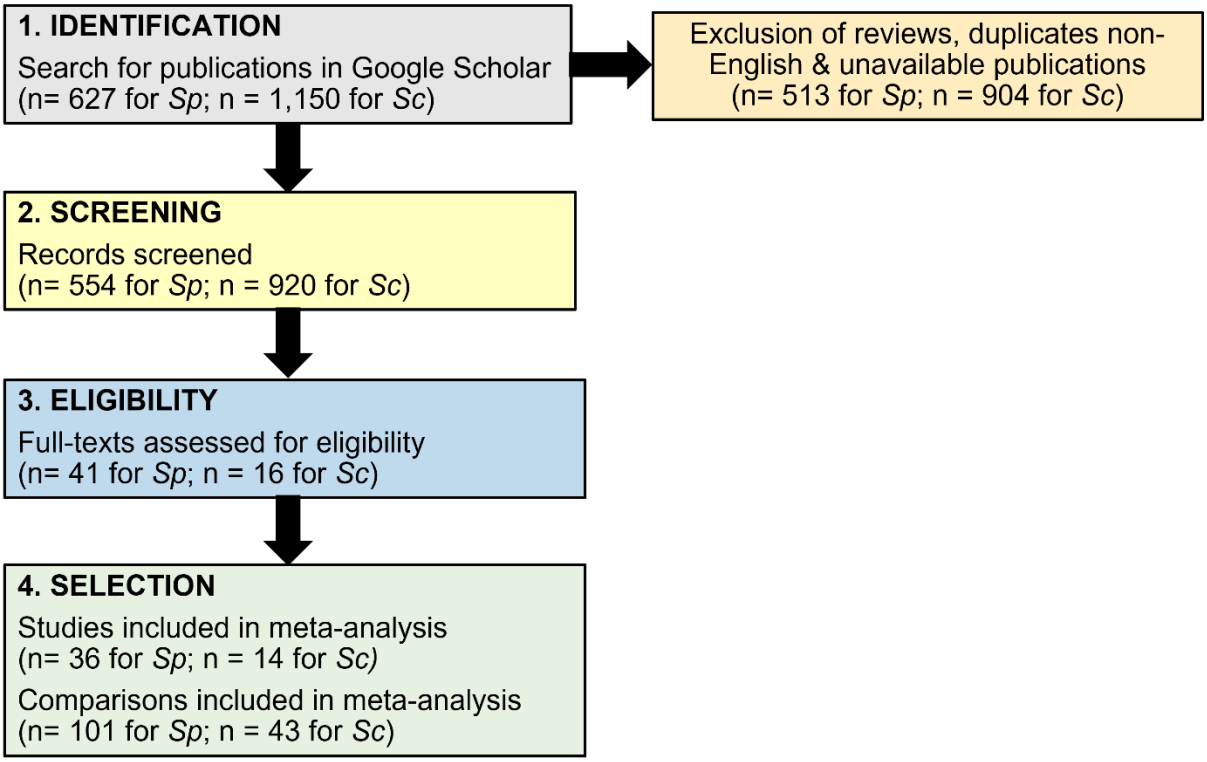


Figure 2.1. PRISMA workflow used to select publications for inclusion in the meta-analysis of nutritional benefits of microalgae in fish. Sp= Spirulina dataset, Sc= Schizochytrium dataset.

2.2.2 Data extraction

The following data were obtained from the selected papers: (1) first author and year of publication, (2) mean value of the standard growth rate (SGR, for the *Spirulina* dataset) or omega-3 content of the fillet (for the *Schizochytrium* dataset) for the treatment (M*e*) and control groups (M*c*), (3) standard deviations of M*e* and M*c*, denoted as S*e* or S*c*, (4) number of fish sampled from the treatment (N*e*) and control (N*c*) groups; when fish were sampled as a batch each batch counted as one sample only, (5) scientific name and family, (6) habitat (freshwater - FW; saltwater - SW), (7) diet (carnivorous, C; omnivorous, O; herbivorous, H), (8) initial mass of the fish (g), (9) replacement level, expressed as %, of fish meal or fish oil and plant oil replaced with microalgae, (10) duration of the trial (days), (11) level of replication (number of tanks); (12) fish density (No. fish per tank) and (13) type of data (data obtained from individual fish, or pooled from batch measurements).

2.2.3 Data analysis

We used R v3.5.1. (R Core Team 2020) for all statistical analysis. Study effect sizes were calculated as standardized mean differences (SMD) between the micro-algae enriched diet and the control diet (without micro-algae) adjusted for small sample size via Hedges' *g* correction (Hedges and Olkin 2014). After inspection of the data, a random effects model was chosen to derive the overall effect (Borenstein, Hedges et al. 2011), since a single underlying common effect (fixed effect model) could not be assumed. Although a random effects model will have wider confidence intervals than a model that assumes a common fixed effect, it is more realistic and also enabled us to examine how effect sizes varied across populations (Borenstein 2019). To fit the random model, we used the in between-study-variance HKSJ estimator method (Hartung and Knapp 2001) in the *meta* and *metafor* R packages. Forest plots were used to visualize the outputs of the meta-analysis.

We examined three measures of heterogeneity among studies: Cohran's Q, with a cut-off of $p = 0.10$ (Higgins, Thompson et al. 2003), the I^2 index which varies from <25% to >75% for small and substantial levels of heterogeneity, respectively (Higgins, Thompson et al. 2003), and tau-squared (τ^2), which represents the between-study variance (Higgins, Deeks et al. 2008). Evidence for publication bias was assessed by inspection of funnel plots (Peters, Sutton et al. 2008), followed by Egger's linear regression test of funnel plot asymmetry (Egger, Smith et al. 1997) and by the p-curve method (Harrer, Cuijpers et al. 2021). Funnel plots compare the observed distribution of effect sizes on the x-axis against their standard error on the y-axis, which is typically inverted. In the absence of publication bias, studies should be contained

within a symmetrical funnel at both sides of the pooled effect size. Studies that lie outside the funnel might indicate the existence of publication bias, although high heterogeneity can also result in asymmetrical funnels (Harrer, Cuijpers et al. 2021). The *p*-curve method compares the significance level of the significant effect sizes against a theoretical left skewed and flat distributions, on the assumption that the most significant results should also be the rarest (Simonsohn, Nelson et al. 2014, Simonsohn, Nelson et al. 2014). It can be used as a diagnostic tool for assessing the presence of publication bias, although it is also affected by high study heterogeneity (Harrer, Cuijpers et al. 2021), and is most useful when heterogeneity is small to moderate (i.e. I^2 <50%).

Dose-effects (i.e. to what extent the nutritional benefits of micro-algae depended on replacement levels) were assessed via mixed-effects meta-regression with the Sidik-Jonkman estimator for τ^2 in the *dmetar* R package, using replacement, fish size, family and habitat as predictors. Inspection of AIC values was used to arrive at the minimal adequate model.

2.2.4. Outlier detection

We employed two methods to detect potential outliers and overly influential studies using the *dmetar* R package (Harrer, Cuijpers et al. 2021): the '*find.outliers*' function using a random effects model and the use of "baujat" plots to help identify studies with a large overall contribution to the overall heterogeneity and a large influence on the pooled results (Baujat, Mahé et al. 2002). Models were refitted after exclusion of outliers and overly influential points.

2.2.5 Subgroup analysis

To gain insights into potential sources of variation in the benefits of using microalgae we carried out a subgroup analysis according to fish family, habitat (freshwater or marine), broad diet guild (carnivore, omnivore, herbivore) and type of measurements (data collected from individual fish, or pooled from a batch).

2.4 Results

2.3.1. Effects of *Spirulina* replacement on Standard Growth rate (SGR)

We found 36 quantitative studies on the effects of *Spirulina* replacement on fish growth *Spirulina* (representing k = 101 control-treatment comparisons) that met the selection criteria. These were carried out in 17 species belonging to 11 different fish families, mostly juveniles

(weight range = 0.02 - 131g) living in freshwater (88%), and having a plant or omnivore diet, like tilapia (*Oreochromis* sp. – 37% of studies), various cyprinids (10% of studies) and catfishes (8% of studies). In most cases, studies were carried out in triplicate tanks and involved an average of 34 individuals per tank (SD = 62), with feeding trials typically lasting between 70 and 120 days (**Table 2.1**). Replacement levels of *Spirulina* varied from 0.025% to 45% (mean = 8.9%, SD = 9.9).

Table 2.1. Results of feeding studies assessing the effects of Spirulina replacement on Standard Growth Rate (SGR, %) of farmed fish.

Author	Study ID	SMD	Me	Se	Mc	Sc	Ne	Nc	`Species	Family	Water	Diet	Size (g)	Replac. %	Days	Tanks	Dens.	Data
El-Sheekh.2014	s1	0.3711	4.69	0.27	4.59	0.27	30	30	*O niloticus x O mossambicus*	Cichlidae	FW	H	0.206	14.00	65	3	10	Indiv
El-Sheekh.2014	s1	3.6974	5.30	0.03	4.59	0.27	30	30	*O niloticus x O mossambicus*	Cichlidae	FW	H	0.206	22.50	65	3	10	Indiv
El-Sheekh.2014	s1	1.4373	4.90	0.14	4.59	0.27	30	30	*O niloticus x O mossambicus*	Cichlidae	FW	H	0.206	28.00	65	3	10	indiv.
Abdel-Latif.2014	s2	3.7009	0.16	0.04	0.04	0.00	20	20	*Oreochromis niloticus*	Cichlidae	FW	H	50.00	10.00	28	1	20	indiv.
Abdel-Latif.2014	s2	1.8504	0.10	0.04	0.04	0.00	20	20	*Oreochromis niloticus*	Cichlidae	FW	H	50.00	2.500	28	1	20	indiv.
Abdel-Latif.2014	s2	2.4673	0.12	0.04	0.04	0.00	20	20	*Oreochromis niloticus*	Cichlidae	FW	H	50.00	5.000	28	1	20	indiv.
Abdel-Tawwab.2009	s3	0.7812	2.62	0.10	2.41	0.36	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	1.880	0.500	84	NA	20	indiv.
Abdel-Tawwab.2009	s3	0.1351	2.45	0.20	2.41	0.36	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	1.880	0.125	84	NA	20	indiv.
Abdel-Tawwab.2009	s3	0.2653	2.49	0.22	2.41	0.36	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	1.880	0.250	84	NA	20	indiv.
Abdel-Tawwab.2009	s3	0.1092	2.45	0.36	2.41	0.36	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	1.880	0.750	84	NA	20	indiv.
Abdel-Tawwab.2009	s3	0.0793	2.44	0.39	2.41	0.36	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	1.880	1.000	84	NA	20	indiv.
Belal.2012	s4	0.3523	2.05	0.51	1.87	0.51	40	40	*Oreochromis niloticus*	Cichlidae	FW	H	7.080	1.000	84	3	10	indiv.
Hussein.2013	s5	6.3402	5.85	0.08	5.04	0.16	39	39	*Oreochromis niloticus*	Cichlidae	FW	H	0.020	43.63	77	3	50	batch
Mahmoud.2018	s6	0.2032	0.82	0.23	0.78	0.15	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	9.300	1.000	83	3	20	indiv.
Mahmoud.2018	s6	-0.4162	0.73	0.08	0.78	0.15	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	9.300	2.000	83	3	20	indiv.
Khalila.2018	s7	0.2969	1.80	0.38	1.70	0.28	90	90	*Oreochromis niloticus*	Cichlidae	FW	H	3.780	0.500	84	3	10	indiv.
Hussein.2014	s8	4.4047	4.20	0.20	3.50	0.10	75	75	*Oreochromis niloticus*	Cichlidae	FW	H	30.00	21.80	63	3	50	indiv.
Leite.2019	s9	-1.5181	4.57	0.32	5.12	0.31	4	4	*Oreochromis niloticus*	Cichlidae	SW	H	1.000	20.00	45	2	25	batch
Teuling.2017	s10	0.4988	2.64	0.37	2.41	0.37	3	3	*Oreochromis niloticus*	Cichlidae	FW	H	37.40	30.00	33	3	35	batch
Liu.2019	s11	0.1953	2.57	0.61	2.36	1.35	15	15	*Pelteobagrus fulvidraco*	Bagridae	FW	C	3.100	5.700	50	3	50	batch
Liu.2019	s11	-0.0359	2.32	0.73	2.36	1.35	15	15	*Pelteobagrus fulvidraco*	Bagridae	FW	C	3.100	11.50	50	3	50	batch
Liu.2019	s11	-0.0066	2.35	1.59	2.36	1.35	15	15	*Pelteobagrus fulvidraco*	Bagridae	FW	C	3.100	17.20	50	3	50	batch
Liu.2019	s11	-0.0388	2.29	2.08	2.36	1.35	15	15	*Pelteobagrus fulvidraco*	Bagridae	FW	C	3.100	23.00	50	3	50	batch
Liu.2019	s11	-0.4739	1.81	0.86	2.36	1.35	15	15	*Pelteobagrus fulvidraco*	Bagridae	FW	C	3.100	28.70	50	3	50	batch
Yu.2018	s12	7.0039	1.69	0.05	1.40	0.03	90	90	*Plectropomus leopardus*	Serranidae	SW	C	18.00	10.00	56	3	30	indiv.
Yu.2018	s12	0.3906	1.41	0.02	1.40	0.03	90	90	*Plectropomus leopardus*	Serranidae	SW	C	18.00	2.000	56	3	30	indiv.
Yu.2018	s12	0.8241	1.45	0.08	1.40	0.03	90	90	*Plectropomus leopardus*	Serranidae	SW	C	18.00	4.000	56	3	30	indiv.
Yu.2018	s12	0.2415	1.41	0.05	1.40	0.03	90	90	*Plectropomus leopardus*	Serranidae	SW	C	18.00	6.000	56	3	30	indiv.
Yu.2018	s12	3.7787	1.58	0.06	1.40	0.03	90	90	*Plectropomus leopardus*	Serranidae	SW	C	18.00	8.000	56	3	30	indiv.
Rosas.2019	s13	12.3432	4.32	0.08	3.38	0.07	30	30	*Mugil liza*	Mugilidae	SW	O	0.470	22.50	90	4	25	indiv.
Rosas.2019	s13	15.9283	4.30	0.04	3.38	0.07	30	30	*Mugil liza*	Mugilidae	SW	O	0.470	15.00	90	4	25	indiv.
Rosas.2019	s13	8.1385	4.19	0.12	3.38	0.07	30	30	*Mugil liza*	Mugilidae	SW	O	0.470	30.00	90	4	25	indiv.
Rosas.2019	s13	3.3596	3.89	0.20	3.38	0.07	30	30	*Mugil liza*	Mugilidae	SW	O	0.470	45.00	90	4	25	indiv.
Adel.2016	s14	3.7430	2.78	0.19	2.22	0.09	60	60	*Huso huso*	Acipenseridae	FW	C	32.16	10.00	56	3	20	indiv.
Adel.2016	s14	1.1242	2.34	0.12	2.22	0.09	60	60	*Huso huso*	Acipenseridae	FW	C	32.16	2.500	56	3	20	indiv.

Adel.2016	s14	2.8706	2.56	0.14	2.22	0.09	60	60	*Huso huso*	Acipenseridae	FW	C	32.16	5.000	56	3	20	indiv.
Cao.2018	s15	0.7200	1.52	0.24	1.37	0.16	66	66	*Carassius auratus gibelio*	Cyprinidae	FW	O	15.37	3.380	46	3	22	indiv.
Cao.2018	s15	0.6240	1.50	0.24	1.37	0.16	66	66	*Carassius auratus gibelio*	Cyprinidae	FW	O	15.37	6.760	46	3	22	indiv.
Cao.2018	s15	-0.1935	1.32	0.32	1.37	0.16	66	66	*Carassius auratus gibelio*	Cyprinidae	FW	O	15.37	13.52	46	3	22	indiv.
Rosas.2019	s16	0.4198	3.75	0.12	3.36	1.30	42	42	*Mugil liza*	Mugilidae	SW	O	0.260	1.950	80	3	14	indiv.
Rosas.2019	s16	0.1501	3.50	0.17	3.36	1.30	42	42	*Mugil liza*	Mugilidae	SW	O	0.260	1.200	80	3	14	indiv.
Rosas.2019	s16	0.0841	3.44	0.31	3.36	1.30	42	42	*Mugil liza*	Mugilidae	SW	O	0.260	2.700	80	3	14	indiv.
Rosas.2019	s16	-0.7006	2.70	0.25	3.36	1.30	42	42	*Mugil liza*	Mugilidae	SW	O	0.260	3.900	80	3	14	indiv.
Ribeiro.2019	s17	1.5381	3.79	0.22	3.43	0.24	24	24	*C macropomum P brachypomus*	Serrasalmidae	FW	H	3.560	40.00	64	3	8	indiv.
Ribeiro.2019	s17	0.9435	3.67	0.26	3.43	0.24	24	24	*C macropomum P brachypomus*	Serrasalmidae	FW	H	3.560	20.00	64	3	8	indiv.
Nasir.2018	s18	0.4371	4.38	0.47	4.15	0.57	90	90	*Clarias gariepinus*	Clariidae	FW	O	2.620	3.000	90	3	30	indiv.
Nasir.2018	s18	0.0805	4.20	0.66	4.15	0.57	90	90	*Clarias gariepinus*	Clariidae	FW	O	2.620	1.000	90	3	30	indiv.
Nasir.2018	s18	0.1749	4.25	0.57	4.15	0.57	90	90	*Clarias gariepinus*	Clariidae	FW	O	2.620	5.000	90	3	30	indiv.
Nasir.2018	s18	0.3381	4.36	0.66	4.15	0.57	90	90	*Clarias gariepinus*	Clariidae	FW	O	2.620	7.000	90	3	30	indiv.
Chainapong.2018	s19	0.0084	1.82	1.59	1.80	2.94	30	30	*Clarias macrocephalus*	Clariidae	FW	O	19.00	10.00	120	3	50	batch
Chainapong.2018	s19	-0.0254	1.70	4.65	1.80	2.94	30	30	*Clarias macrocephalus*	Clariidae	FW	O	19.00	5.000	120	3	50	batch
El-Ward.2016	s20	5.6908	1.87	0.11	1.24	0.11	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	9.900	10.89	56	3	20	indiv.
El-Ward.2016	s20	-0.7769	1.15	0.12	1.24	0.11	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	9.900	2.730	56	3	20	indiv.
El-Ward.2016	s20	1.2087	1.40	0.15	1.24	0.11	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	9.900	5.450	56	3	20	indiv.
El-Ward.2016	s20	4.1041	1.76	0.14	1.24	0.11	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	9.900	8.170	56	3	20	indiv.
Zeinab.2019	s21	0.2463	2.32	0.40	2.22	0.40	45	45	*Oreochromis niloticus*	Cichlidae	FW	H	2.700	3.000	95	3	15	indiv.
Zeinab.2019	s21	0.0000	2.22	0.40	2.22	0.40	45	45	*Oreochromis niloticus*	Cichlidae	FW	H	2.700	5.000	95	3	15	indiv.
Zeinab.2019	s21	-0.2463	2.12	0.40	2.22	0.40	45	45	*Oreochromis niloticus*	Cichlidae	FW	H	2.700	7.000	95	3	15	indiv.
Teimouri.2016	s22	0.2638	1.39	0.30	1.31	0.30	36	36	*Oncorhynchus mykiss*	Salmonidae	FW	C	101.0	7.500	70	3	12	indiv.
Teimouri.2016	s22	0.0800	1.33	0.18	1.31	0.30	36	36	*Oncorhynchus mykiss*	Salmonidae	FW	C	101.0	2.500	70	3	12	indiv.
Teimouri.2016	s22	-0.1999	1.26	0.18	1.31	0.30	36	36	*Oncorhynchus mykiss*	Salmonidae	FW	C	101.0	5.000	70	3	12	indiv.
Teimouri.2016	s22	0.1977	1.39	0.48	1.31	0.30	36	36	*Oncorhynchus mykiss*	Salmonidae	FW	C	101.0	10.00	70	3	12	indiv.
Güroy.2019	s23	-2.7770	0.81	0.05	0.98	0.07	60	60	*Oncorhynchus mykiss*	Salmonidae	FW	C	135.0	4.000	84	3	20	indiv.
El-Murr.2014	s24	0.5289	0.57	0.39	0.40	0.23	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	33.00	1.500	60	3	50	indiv.
El-Murr.2014	s24	0.2177	0.46	0.31	0.40	0.23	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	33.00	0.500	60	3	50	indiv.
El-Murr.2014	s24	0.4978	0.56	0.39	0.40	0.23	60	60	*Oreochromis niloticus*	Cichlidae	FW	H	33.00	1.000	60	3	50	indiv.
Al-Zayat.2019	s25	4.3783	1.26	0.05	1.05	0.04	20	20	*Oreochromis niloticus*	Cichlidae	FW	H	6.000	0.750	60	2	10	indiv.
Al-Zayat.2019	s25	0.2255	1.08	0.18	1.05	0.04	20	20	*Oreochromis niloticus*	Cichlidae	FW	H	6.000	0.250	60	2	10	indiv.
Al-Zayat.2019	s25	1.5341	1.12	0.04	1.05	0.04	20	20	*Oreochromis niloticus*	Cichlidae	FW	H	6.000	0.500	60	2	10	indiv.
Roohani.2019	s26	0.4656	1.58	1.75	0.81	1.42	12	12	*Salmo trutta caspius*	Salmonidae	FW	C	11.00	3.960	70	3	40	batch
Roohani.2019	s26	0.2204	1.10	1.10	0.81	1.42	12	12	*Salmo trutta caspius*	Salmonidae	FW	C	11.00	1.320	70	3	40	batch
Roohani.2019	s26	0.2466	1.16	1.31	0.81	1.42	12	12	*Salmo trutta caspius*	Salmonidae	FW	C	11.00	2.640	70	3	40	batch
Roohani.2019	s26	0.2487	1.39	2.85	0.81	1.42	12	12	*Salmo trutta caspius*	Salmonidae	FW	C	11.00	5.280	70	3	40	batch
Kermani.2020	s27	1.9740	3.10	0.10	2.90	0.10	30	30	*Oncorhynchus mykiss*	Salmonidae	FW	C	12.60	0.025	56	3	10	indiv.

Kermani.2020	s27	0.0000	2.90	0.50	2.90	0.10	30	30	*Oncorhynchus mykiss*	Salmonidae	FW	C	12.60	0.050	56	3	10	indiv.
Kermani.2020	s27	-0.6242	2.80	0.20	2.90	0.10	30	30	*Oncorhynchus mykiss*	Salmonidae	FW	C	12.60	0.100	56	3	10	indiv.
Kermani.2020	s27	-1.9740	2.70	0.10	2.90	0.10	30	30	*Oncorhynchus mykiss*	Salmonidae	FW	C	12.60	0.250	56	3	10	indiv.
Gouveia.2003 (koicarp)	s28	0.0000	0.20	0.10	0.20	0.10	25	25	*Cyprinus carpio*	Cyprinidae	FW	O	24.60	4.000	70	2	25	indiv.
Gouveia.2003 (goldfish)	s29	0.0000	1.40	0.04	1.40	0.09	25	25	*Carassius auratus*	Cyprinidae	FW	O	0.900	4.000	70	2	25	indiv.
Abdel-Warith.2019	s30	-0.0322	1.89	0.73	1.91	0.47	30	30	*Oreochromis niloticus*	Cichlidae	FW	H	15.98	4.000	84	2	15	indiv.
Abdel-Warith.2019	s30	-0.2929	1.80	0.23	1.91	0.47	30	30	*Oreochromis niloticus*	Cichlidae	FW	H	15.98	8.000	84	2	15	indiv.
Abdel-Warith.2019	s30	-0.1902	1.83	0.35	1.91	0.47	30	30	*Oreochromis niloticus*	Cichlidae	FW	H	15.98	12.00	84	2	15	indiv.
Khanzadeh.2016	s31	0.6901	2.31	0.10	2.20	0.20	48	48	*Trichopodus trichopterus*	Osphronemidae	FW	H	1.290	10.00	112	3	48	indiv.
Khanzadeh.2016	s31	-0.2480	2.15	0.20	2.20	0.20	48	48	*Trichopodus trichopterus*	Osphronemidae	FW	H	1.290	2.500	112	3	48	indiv.
Khanzadeh.2016	s31	0.0778	2.22	0.30	2.20	0.20	48	48	*Trichopodus trichopterus*	Osphronemidae	FW	H	1.290	5.000	112	3	48	indiv.
Khanzadeh.2016	s31	0.2480	2.25	0.20	2.20	0.20	48	48	*Trichopodus trichopterus*	Osphronemidae	FW	H	1.290	20.00	112	3	48	indiv.
Raji.2019	s32	0.3338	2.29	0.09	2.26	0.09	80	80	*Clarias gariepinus*	Clariidae	FW	O	41.86	18.70	56	3	10	indiv.
Raji.2019	s32	0.2225	2.28	0.09	2.26	0.09	80	80	*Clarias gariepinus*	Clariidae	FW	O	41.86	12.50	56	3	10	indiv.
Viswanathan.2019	s33	0.1114	1.90	2.00	1.70	1.50	25	25	*Cyprinus carpio*	Cyprinidae	FW	O	21.50	15.00	28	3	25	indiv.
Viswanathan.2019	s33	0.0772	1.80	1.00	1.70	1.50	25	25	*Cyprinus carpio*	Cyprinidae	FW	O	21.50	5.000	28	3	25	indiv.
Viswanathan.2019	s33	0.0656	1.80	1.50	1.70	1.50	25	25	*Cyprinus carpio*	Cyprinidae	FW	O	21.50	10.00	28	3	25	indiv.
Viswanathan.2019	s33	0.0656	1.80	1.50	1.70	1.50	25	25	*Cyprinus carpio*	Cyprinidae	FW	O	21.50	20.00	28	3	25	indiv.
Viswanathan.2019	s33	0.0000	1.70	1.00	1.70	1.50	25	25	*Cyprinus carpio*	Cyprinidae	FW	O	21.50	25.00	28	3	25	indiv.
Kim.2013	s34	4.4363	0.81	0.01	0.68	0.04	75	75	*Oplegnathus fasciatus*	Oplegnathidae	SW	H	57.00	9.000	56	3	25	indiv.
Kim.2013	s34	1.8877	0.74	0.02	0.68	0.04	75	75	*Oplegnathus fasciatus*	Oplegnathidae	SW	H	57.00	18.00	56	3	25	indiv.
Kim.2013	s34	0.1951	0.69	0.06	0.68	0.04	75	75	*Oplegnathus fasciatus*	Oplegnathidae	SW	H	57.00	26.00	56	3	25	indiv.
Rosas.2019	s35	6.3017	4.21	0.17	3.38	0.07	30	30	*Mugil liza*	Mugilidae	SW	O	0.470	4.000	75	3	10	indiv.
Rosas.2019	s35	6.1543	4.15	0.16	3.38	0.07	30	30	*Mugil liza*	Mugilidae	SW	O	0.470	2.000	75	3	10	indiv.
Siringi.2007	s36	2.1807	0.52	0.08	0.35	0.08	60	60	*Oreochromis shiranus*	Cichlidae	FW	H	5.860	0.700	70	3	20	indiv.
Siringi.2007	s36	1.0262	0.43	0.08	0.35	0.08	60	60	*Oreochromis shiranus*	Cichlidae	FW	H	5.860	0.350	70	3	20	indiv.
Siringi.2007	s36	1.0547	0.48	0.15	0.35	0.08	60	60	*Oreochromis shiranus*	Cichlidae	FW	H	5.860	1.050	70	3	20	indiv.

SMD = Standardized Mean Difference; Me = Mean SGR of experimental group; Se = standard deviation of SGR of experimental group; Mc = Mean SGR of control group; Sc = standard deviation of SGR of control group; Ne = sample size of experimental group; Nc = sample size of control group; FW = freshwater; SW = sea water; Diet = Carnivore (C), Herbivore (H), Omnivore (O); Size= Initial mass (g); Replac. % = Spirulina replacement level (%); Days = duration of trial (days); Tanks = Number of replicate tanks; Dens. = Tank density (No. fish/tank); Data = type of measurement (individual weights or batch weighing).

Effect sizes

Standardized mean differences (SMD), corrected for small sample sizes, varied between -2.78 and 15.93. The pooled SMD of the random effects model was 1.21 (95%CI = 0.71-1.70) which was significantly different from zero ($t = 4.83$, $P < 0.001$), and indicated that *Spirulina* inclusion in the diet had a positive effect on fish growth (**Figure 2.2**). However, heterogeneity between studies was very high ($Q = 2732$, $df = 100$, $P < 0.001$; $I^2 = 96.3\%$ $\tau^2 = 6.26$) and the prediction interval was wide (95% CI = -3.78; 6.16), indicating that negative effects on growth cannot be ruled out in future studies. Just over 48% of control-treatment comparisons (49/101) were statistically significant, involving 19 of the 36 independent studies (53%). The average replacement of fish meal with *Spirulina* that yielded an improvement in SGR was 8.42% (SD = 10.26), but enhanced growth was detected with *Spirulina* replacement as low as 0.025% in rainbow trout (*Oncorhynchus mykiss*)(Kermani, Babaei et al. 2020).

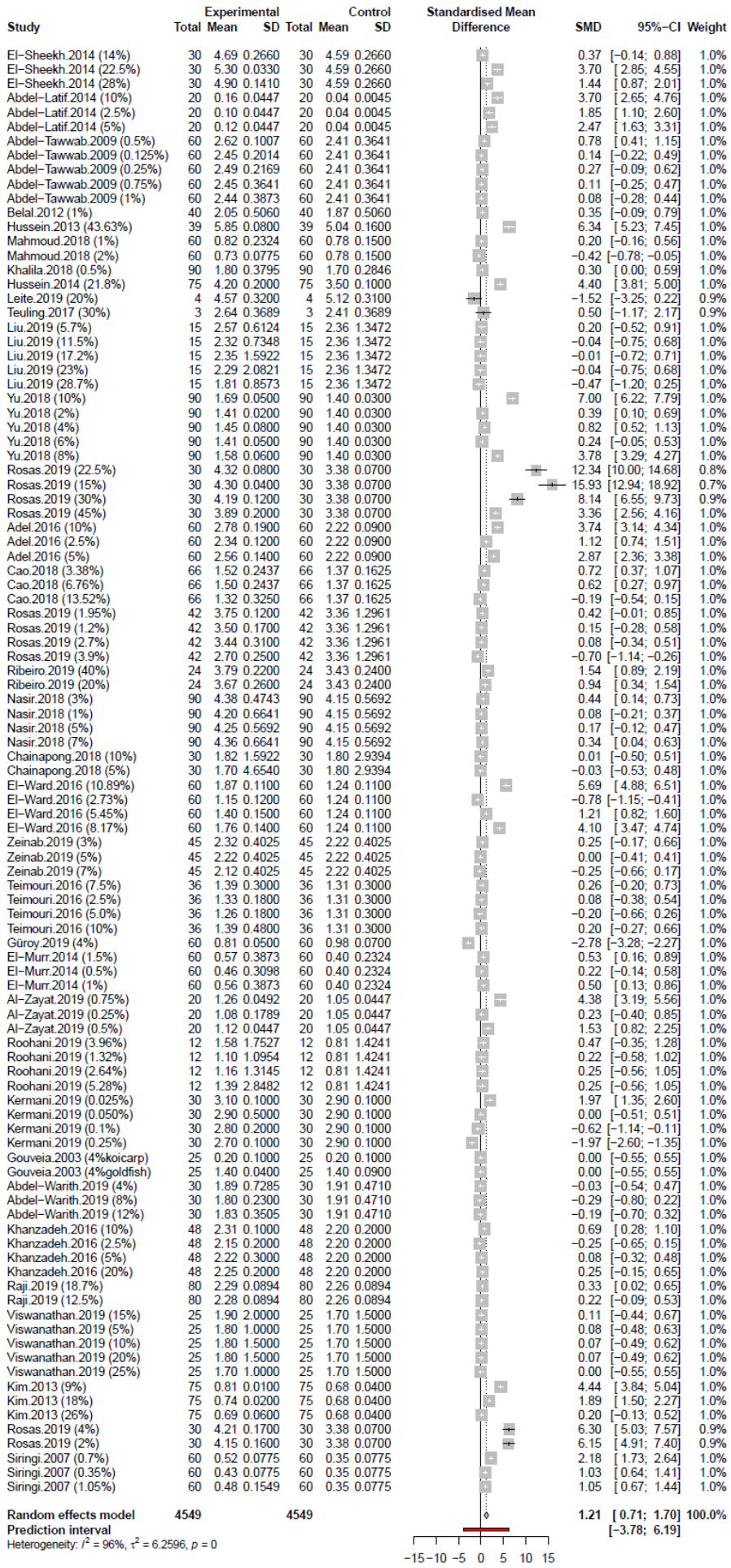

Experimental
Control
Standardised Mean Difference
Study Total Mean SD Total Mean SD SMD 95%−CI Weight
El−Sheekh.2014 (14%) 30 4.69 0.2660 30 4.59 0.2660 0.37 [−0.14; 0.88] 1.0%
El−Sheekh.2014 (22.5%) 30 5.30 0.0330 30 4.59 0.2660 3.70 [2.85; 4.55] 1.0%
El−Sheekh.2014 (28%) 30 4.90 0.1410 30 4.59 0.2660 1.44 [0.87; 2.01] 1.0%
Abdel−Latif.2014 (10%) 20 0.16 0.0447 20 0.04 0.0045 3.70 [2.65; 4.76] 1.0%
Abdel−Latif.2014 (2.5%) 20 0.10 0.0447 20 0.04 0.0045 1.85 [1.10; 2.60] 1.0%
Abdel−Latif.2014 (5%) 20 0.12 0.0447 20 0.04 0.0045 2.47 [1.63; 3.31] 1.0%
Abdel−Tawwab.2009 (0.5%) 60 2.62 0.1007 60 2.41 0.3641 0.78 [0.41; 1.15] 1.0%
Abdel−Tawwab.2009 (0.125%) 60 2.45 0.2014 60 2.41 0.3641 0.14 [−0.22; 0.49] 1.0%
Abdel−Tawwab.2009 (0.25%) 60 2.49 0.2169 60 2.41 0.3641 0.27 [−0.09; 0.62] 1.0%
Abdel−Tawwab.2009 (0.75%) 60 2.45 0.3641 60 2.41 0.3641 0.11 [−0.25; 0.47] 1.0%
Abdel−Tawwab.2009 (1%) 60 2.44 0.3873 60 2.41 0.3641 0.08 [−0.28; 0.44] 1.0%
Belal.2012 (1%) 40 2.05 0.5060 40 1.87 0.5060 0.35 [−0.09; 0.79] 1.0%
Hussein.2013 (43.63%) 39 5.85 0.0800 39 5.04 0.1600 6.34 [5.23; 7.45] 1.0%
Mahmoud.2018 (1%) 60 0.82 0.2324 60 0.78 0.1500 0.20 [−0.16; 0.56] 1.0%
Mahmoud.2018 (2%) 60 0.73 0.0775 60 0.78 0.1500 −0.42 [−0.78; −0.05] 1.0%
Khalila.2018 (0.5%) 90 1.80 0.3795 90 1.70 0.2846 0.30 [0.00; 0.59] 1.0%
Hussein.2014 (21.8%) 75 4.20 0.2000 75 3.50 0.1000 4.40 [3.81; 5.00] 1.0%
Leite.2019 (20%) 4 4.57 0.3200 4 5.12 0.3100 −1.52 [−3.25; 0.22] 0.9%
Teuling.2017 (30%) 3 2.64 0.3689 3 2.41 0.3689 0.50 [−1.17; 2.17] 0.9%
Liu.2019 (5.7%) 15 2.57 0.6124 15 2.36 1.3472 0.20 [−0.52; 0.91] 1.0%
Liu.2019 (11.5%) 15 2.32 0.7348 15 2.36 1.3472 −0.04 [−0.75; 0.68] 1.0%
Liu.2019 (17.2%) 15 2.35 1.5922 15 2.36 1.3472 −0.01 [−0.72; 0.71] 1.0%
Liu.2019 (23%) 15 2.29 2.0821 15 2.36 1.3472 −0.04 [−0.75; 0.68] 1.0%
Liu.2019 (28.7%) 15 1.81 0.8573 15 2.36 1.3472 −0.47 [−1.20; 0.25] 1.0%
Yu.2018 (10%) 90 1.69 0.0500 90 1.40 0.0300 7.00 [6.22; 7.79] 1.0%
Yu.2018 (2%) 90 1.41 0.0200 90 1.40 0.0300 0.39 [0.10; 0.69] 1.0%
Yu.2018 (4%) 90 1.45 0.0800 90 1.40 0.0300 0.82 [0.52; 1.13] 1.0%
Yu.2018 (6%) 90 1.41 0.0500 90 1.40 0.0300 0.24 [−0.05; 0.53] 1.0%
Yu.2018 (8%) 90 1.58 0.0600 90 1.40 0.0300 3.78 [3.29; 4.27] 1.0%
Rosas.2019 (22.5%) 30 4.32 0.0800 30 3.38 0.0700 12.34 [10.00; 14.68] 0.8%
Rosas.2019 (15%) 30 4.30 0.0400 30 3.38 0.0700 15.93 [12.94; 18.92] 0.7%
Rosas.2019 (30%) 30 4.19 0.1200 30 3.38 0.0700 8.14 [6.55; 9.73] 0.9%
Rosas.2019 (45%) 30 3.89 0.2000 30 3.38 0.0700 3.36 [2.56; 4.16] 1.0%
Adel.2016 (10%) 60 2.78 0.1900 60 2.22 0.0900 3.74 [3.14; 4.34] 1.0%
Adel.2016 (2.5%) 60 2.34 0.1200 60 2.22 0.0900 1.12 [0.74; 1.51] 1.0%
Adel.2016 (5%) 60 2.56 0.1400 60 2.22 0.0900 2.87 [2.36; 3.38] 1.0%
Cao.2018 (3.38%) 66 1.52 0.2437 66 1.37 0.1625 0.72 [0.37; 1.07] 1.0%
Cao.2018 (6.76%) 66 1.50 0.2437 66 1.37 0.1625 0.62 [0.27; 0.97] 1.0%
Cao.2018 (13.52%) 66 1.32 0.3250 66 1.37 0.1625 −0.19 [−0.54; 0.15] 1.0%
Rosas.2019 (1.95%) 42 3.75 0.1200 42 3.36 1.2961 0.42 [−0.01; 0.85] 1.0%
Rosas.2019 (1.2%) 42 3.50 0.1700 42 3.36 1.2961 0.15 [−0.28; 0.58] 1.0%
Rosas.2019 (2.7%) 42 3.44 0.3100 42 3.36 1.2961 0.08 [−0.34; 0.51] 1.0%
Rosas.2019 (3.9%) 42 2.70 0.2500 42 3.36 1.2961 −0.70 [−1.14; −0.26] 1.0%
Ribeiro.2019 (40%) 24 3.79 0.2200 24 3.43 0.2400 1.54 [0.89; 2.19] 1.0%
Ribeiro.2019 (20%) 24 3.67 0.2600 24 3.43 0.2400 0.94 [0.34; 1.54] 1.0%
Nasir.2018 (3%) 90 4.38 0.4743 90 4.15 0.5692 0.44 [0.14; 0.73] 1.0%
Nasir.2018 (1%) 90 4.20 0.6641 90 4.15 0.5692 0.08 [−0.21; 0.37] 1.0%
Nasir.2018 (5%) 90 4.25 0.5692 90 4.15 0.5692 0.17 [−0.12; 0.47] 1.0%
Nasir.2018 (7%) 90 4.36 0.6641 90 4.15 0.5692 0.34 [0.04; 0.63] 1.0%
Chainapong.2018 (10%) 30 1.82 1.5922 30 1.80 2.9394 0.01 [−0.50; 0.51] 1.0%
Chainapong.2018 (5%) 30 1.70 4.6540 30 1.80 2.9394 −0.03 [−0.53; 0.48] 1.0%
El−Ward.2016 (10.89%) 60 1.87 0.1100 60 1.24 0.1100 5.69 [4.88; 6.51] 1.0%
El−Ward.2016 (2.73%) 60 1.15 0.1200 60 1.24 0.1100 −0.78 [−1.15; −0.41] 1.0%
El−Ward.2016 (5.45%) 60 1.40 0.1500 60 1.24 0.1100 1.21 [0.82; 1.60] 1.0%
El−Ward.2016 (8.17%) 60 1.76 0.1400 60 1.24 0.1100 4.10 [3.47; 4.74] 1.0%
Zeinab.2019 (3%) 45 2.32 0.4025 45 2.22 0.4025 0.25 [−0.17; 0.66] 1.0%
Zeinab.2019 (5%) 45 2.22 0.4025 45 2.22 0.4025 0.00 [−0.41; 0.41] 1.0%
Zeinab.2019 (7%) 45 2.12 0.4025 45 2.22 0.4025 −0.25 [−0.66; 0.17] 1.0%
Teimouri.2016 (7.5%) 36 1.39 0.3000 36 1.31 0.3000 0.26 [−0.20; 0.73] 1.0%
Teimouri.2016 (2.5%) 36 1.33 0.1800 36 1.31 0.3000 0.08 [−0.38; 0.54] 1.0%
Teimouri.2016 (5.0%) 36 1.26 0.1800 36 1.31 0.3000 −0.20 [−0.66; 0.26] 1.0%
Teimouri.2016 (10%) 36 1.39 0.4800 36 1.31 0.3000 0.20 [−0.27; 0.66] 1.0%
Güroy.2019 (4%) 60 0.81 0.0500 60 0.98 0.0700 −2.78 [−3.28; −2.27] 1.0%
El−Murr.2014 (1.5%) 60 0.57 0.3873 60 0.40 0.2324 0.53 [0.16; 0.89] 1.0%
El−Murr.2014 (0.5%) 60 0.46 0.3098 60 0.40 0.2324 0.22 [−0.14; 0.58] 1.0%
El−Murr.2014 (1%) 60 0.56 0.3873 60 0.40 0.2324 0.50 [0.13; 0.86] 1.0%
Al−Zayat.2019 (0.75%) 20 1.26 0.0492 20 1.05 0.0447 4.38 [3.19; 5.56] 1.0%
Al−Zayat.2019 (0.25%) 20 1.08 0.1789 20 1.05 0.0447 0.23 [−0.40; 0.85] 1.0%
Al−Zayat.2019 (0.5%) 20 1.12 0.0447 20 1.05 0.0447 1.53 [0.82; 2.25] 1.0%
Roohani.2019 (3.96%) 12 1.58 1.7527 12 0.81 1.4241 0.47 [−0.35; 1.28] 1.0%
Roohani.2019 (1.32%) 12 1.10 1.0954 12 0.81 1.4241 0.22 [−0.58; 1.02] 1.0%
Roohani.2019 (2.64%) 12 1.16 1.3145 12 0.81 1.4241 0.25 [−0.56; 1.05] 1.0%
Roohani.2019 (5.28%) 12 1.39 2.8482 12 0.81 1.4241 0.25 [−0.56; 1.05] 1.0%
Kermani.2019 (0.025%) 30 3.10 0.1000 30 2.90 0.1000 1.97 [1.35; 2.60] 1.0%
Kermani.2019 (0.050%) 30 2.90 0.5000 30 2.90 0.1000 0.00 [−0.51; 0.51] 1.0%
Kermani.2019 (0.1%) 30 2.80 0.2000 30 2.90 0.1000 −0.62 [−1.14; −0.11] 1.0%
Kermani.2019 (0.25%) 30 2.70 0.1000 30 2.90 0.1000 −1.97 [−2.60; −1.35] 1.0%
Gouveia.2003 (4%koicarp) 25 0.20 0.1000 25 0.20 0.1000 0.00 [−0.55; 0.55] 1.0%
Gouveia.2003 (4%goldfish) 25 1.40 0.0400 25 1.40 0.0900 0.00 [−0.55; 0.55] 1.0%
Abdel−Warith.2019 (4%) 30 1.89 0.7285 30 1.91 0.4710 −0.03 [−0.54; 0.47] 1.0%
Abdel−Warith.2019 (8%) 30 1.80 0.2300 30 1.91 0.4710 −0.29 [−0.80; 0.22] 1.0%
Abdel−Warith.2019 (12%) 30 1.83 0.3505 30 1.91 0.4710 −0.19 [−0.70; 0.32] 1.0%
Khanzadeh.2016 (10%) 48 2.31 0.1000 48 2.20 0.2000 0.69 [0.28; 1.10] 1.0%
Khanzadeh.2016 (2.5%) 48 2.15 0.2000 48 2.20 0.2000 −0.25 [−0.65; 0.15] 1.0%
Khanzadeh.2016 (5%) 48 2.22 0.3000 48 2.20 0.2000 0.08 [−0.32; 0.48] 1.0%
Khanzadeh.2016 (20%) 48 2.25 0.2000 48 2.20 0.2000 0.25 [−0.15; 0.65] 1.0%
Raji.2019 (18.7%) 80 2.29 0.0894 80 2.26 0.0894 0.33 [0.02; 0.65] 1.0%
Raji.2019 (12.5%) 80 2.28 0.0894 80 2.26 0.0894 0.22 [−0.09; 0.53] 1.0%
Viswanathan.2019 (15%) 25 1.90 2.0000 25 1.70 1.5000 0.11 [−0.44; 0.67] 1.0%
Viswanathan.2019 (5%) 25 1.80 1.0000 25 1.70 1.5000 0.08 [−0.48; 0.63] 1.0%
Viswanathan.2019 (10%) 25 1.80 1.5000 25 1.70 1.5000 0.07 [−0.49; 0.62] 1.0%
Viswanathan.2019 (20%) 25 1.80 1.5000 25 1.70 1.5000 0.07 [−0.49; 0.62] 1.0%
Viswanathan.2019 (25%) 25 1.70 1.0000 25 1.70 1.5000 0.00 [−0.55; 0.55] 1.0%
Kim.2013 (9%) 75 0.81 0.0100 75 0.68 0.0400 4.44 [3.84; 5.04] 1.0%
Kim.2013 (18%) 75 0.74 0.0200 75 0.68 0.0400 1.89 [1.50; 2.27] 1.0%
Kim.2013 (26%) 75 0.69 0.0600 75 0.68 0.0400 0.20 [−0.13; 0.52] 1.0%
Rosas.2019 (4%) 30 4.21 0.1700 30 3.38 0.0700 6.30 [5.03; 7.57] 0.9%
Rosas.2019 (2%) 30 4.15 0.1600 30 3.38 0.0700 6.15 [4.91; 7.40] 0.9%
Siringi.2007 (0.7%) 60 0.52 0.0775 60 0.35 0.0775 2.18 [1.73; 2.64] 1.0%
Siringi.2007 (0.35%) 60 0.43 0.0775 60 0.35 0.0775 1.03 [0.64; 1.41] 1.0%
Siringi.2007 (1.05%) 60 0.48 0.1549 60 0.35 0.0775 1.05 [0.67; 1.44] 1.0%
Random effects model 4549 4549 1.21 [0.71; 1.70] 100.0%
Prediction interval [−3.78; 6.19]
Heterogeneity: I^2 = 96%, τ^2 = 6.2596, p = 0
−15 −10 −5 0 5 10 15

Figure 2.2. Forest plot summarizing the effect of Spirulina replacement on the Standard Growth Rate (SGR) of farmed finfish. Each trial (n = 101) is represented by a square whose size is proportional to its relative weight, its width represents the 95% CI, the horizontal line the 99% CI, and the center the Standardized Mean Difference (SMD) corrected for small sample size (Hedge's g). The grey diamond at the bottom represents the overall effect extending over the 95% CI. The solid vertical line denotes the zero effect, and the dotted vertical line the SMD under a random effects model.

Dose-effects

Results of meta-regression by mixed-effects modelling indicates that there is a significant positive relationship between *Spirulina* replacement level and standard growth rate while statistically controlling for variation among families ($F_{11,89}$ = 4.629, P < 0.001; **Figure 2.3**). The minimal adequate model included *Spirulina* replacement and family as the only significant predictors of changes in standard growth rate. Initial size (t = -0.685, P = 0.495) and habitat (t = -1.754, P = 0.0829) were not significant and were dropped from the full main effects model. A 1% increase in *Spirulina* inclusion is expected to result in a 0.07% mean increase in SGR (95% CI = 0.03-0.12%), although the model only accounted for 29.4% of the observed heterogeneity and the amount of residual heterogeneity was high (Q_E = 2143.6, df = 89, P < 0.001). Inspection of estimates indicated that negative impacts were also possible. Two families, Bagridae (t = -2.277, P = 0.0250) and Cyprinidae (t = -2.043, P = 0.044) deviated significantly from the general trend and showed a reduction in growth with increasing *Spirulina* replacement levels, while one family, Salmonidae, showed a near significant negative effect (t = -1.909, P = 0.059).

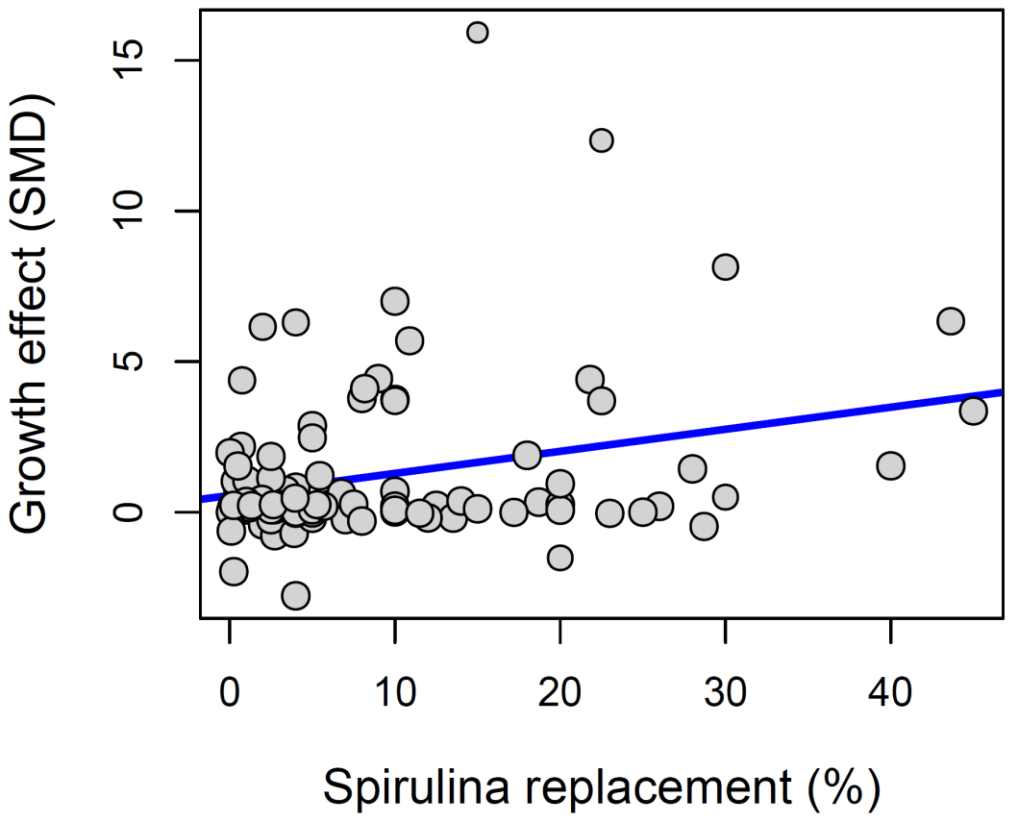


Figure 2.3. Bubble plot showing the estimated regression slope of the meta-regression on the effect of Spirulina replacement (%) on the Standardized Mean Difference in Standard Growth Rate (%). The size of the points is proportional to the weight of each study.

Validity of results

A strong asymmetry was observed in the funnel plot (**Figure 2.4**), which might be indicative of publication bias. Several studies reporting large effects were more precise than one might expect and clustered at the bottom right corner, far outside the boundaries of the funnel. A linear regression test of funnel plot asymmetry (Egger's test) confirmed the observed asymmetry (t_{99} = 5.37, P < 0.001; bias coefficient = 7.04, SE = 1.31). However, caution must be exercised as the high level of heterogeneity in the data set likely also contributed to the asymmetry observed in the funnel plot.

Results from the *p*-curve analysis indicated that the distribution of significant results was significantly right skewed according to all three tests (P binomial < 0.001, full curve P < 0.001; half curve P < 0.001), while results from the flatness test could not reject the hypothesis that the distribution of significant results was dependent on the significance level (P binomial > 0.999, full curve P > 0.999; half curve P > 0.999). Overall, the evidential value suggests that the observed results are driven by a true underlying effect and do not appear to have been affected by publication bias in the form of p-hacking which is the when researchers collect or select data or statistical analyses until nonsignificant results become significant (Head, Holman et al. 2015).

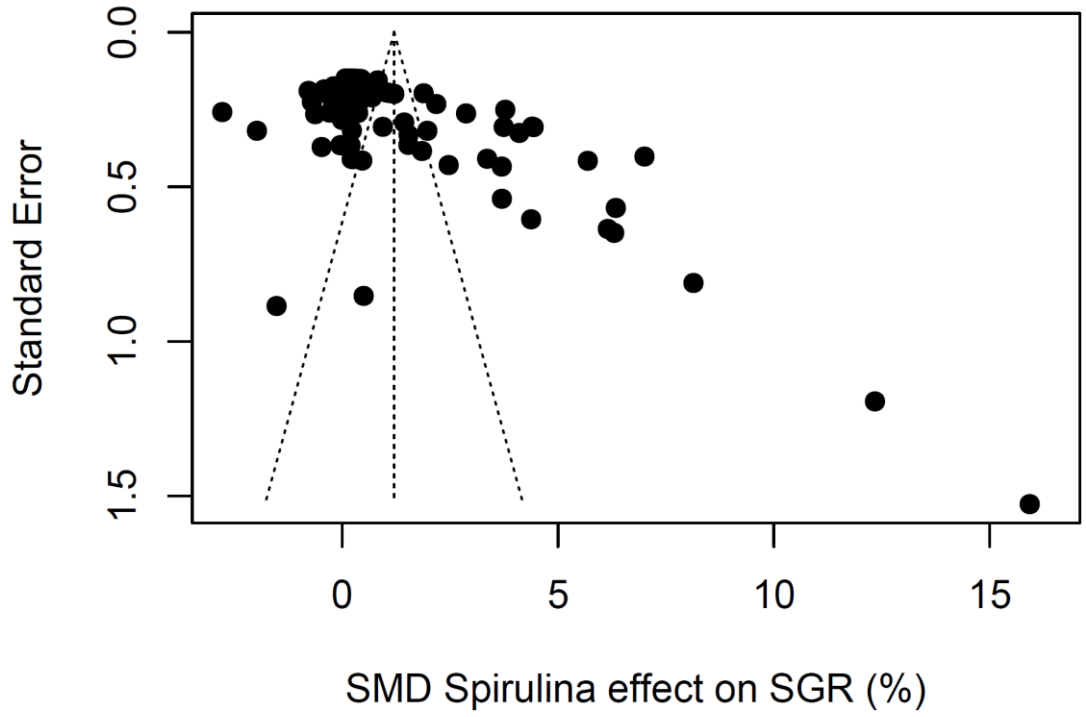


Figure 2.4. Funnel plot showing the relationship between the SMD and the standard error (inverted scale) for the effects of Spirulina replacement on standard growth rate (SGR). Each point represents a treatment-control comparison and the dotted vertical line denotes the global SMD under a random effects model. An asymmetric distribution of points outside the funnel might be indicative of publication bias.

Inspection of Baujat diagnostic plots detected five results which were overly influential (two from the same study) and which also contributed greatly to the overall heterogeneity (**Figure 2.5**), while formal outlier analysis detected 70 extreme results. Reanalysis of the data without the overly influential points resulted in a pooled SMD of 1.09 (95%CI = 0.60-1.57) which is still significantly different from zero (t = 4.44, P < 0.001). Similarly, removal of outliers resulted in a statistically significant SMD of 0.86 (95%CI = 0.64-1.07; t = 8.14, P < 0.001). These results indicate that there is a significant positive effect of *Spirulina* on fish growth which is robust to the presence of extreme values, although heterogeneity even without outliers continues to be high (I^2 = 76.8%, Q = 129.6, df = 30, P < 0.0001) suggesting there are underlying structural differences between studies beyond sampling error.

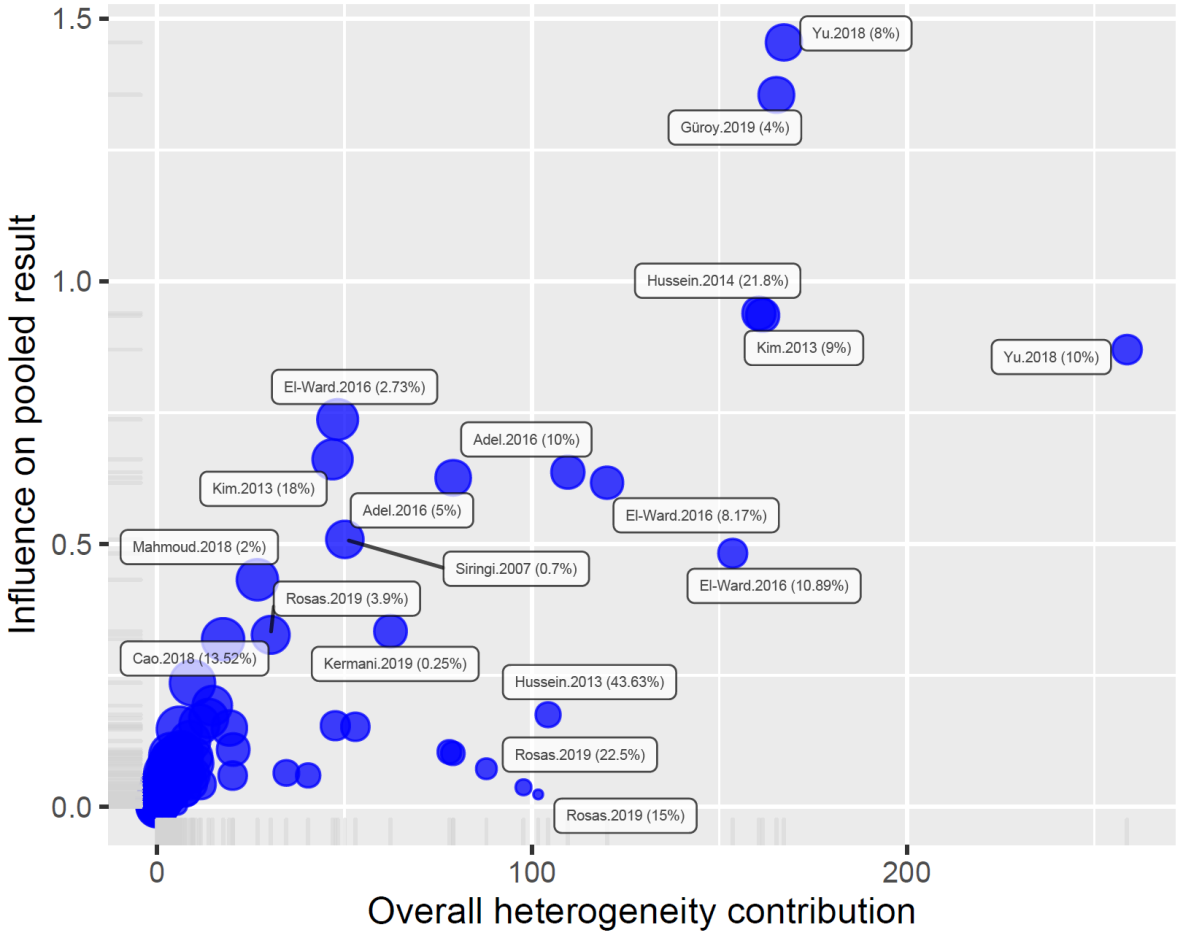


Figure 2.5. Baujat bubble plot used to identify potential outliers in the Spirulina data set, showing the contribution of each study to the overall heterogeneity and its influence under a random effects model. The percentage near the name of the author indicate the microalgal replacement level. The size of each point is proportional to its relative weight in the meta-analysis. Five trials in the upper right corner accounted for a large share of the observed heterogeneity and were also overly influential which merited further scrutiny.

Subgroup analysis

To gain insights into the sources of heterogeneity, results were analysed by different subgroups. Significant differences in *Spirulina* effects were found with respect to fish family (Q = 53.42, df = 10, P < 0.001) and habitat (Q = 7.11, df = 1, P = 0.008), but not with respect to feeding habit (P = 0.305) or type of measurements (P = 0.098; **Table 2.2**). Of the 11 fish families examined, three families (Cichlidae, Clariidae and Mugilidae) showed a statistically significant increase on growth, this effect being strongest for Mugilidae (SMD = 5.11; 95%CI = 1.09- 9.12), followed by Cichlidae (SMD = 1.20; 95%CI = 0.60- 1.80) and Clariidae (SMD = 0.23; 95%CI = 0.10- 0.36).

Differences were also found between freshwater and marine species, both displaying a significant increase in growth following *Spirulina* inclusion, the positive effect on growth being ~5x times greater in marine fish (SMD = 3.56; 95%CI = 1.34- 5.78) than in freshwater fish (SMD = 0.70; 95%CI = 0.38-1.02). Significant *Spirulina* benefits on growth were found for omnivores and herbivores, but not for carnivores. Studies that weighed fish individually were also more likely to reveal a positive effect of *Spirulina* on growth than those which used batch weighing (**Table 2**).

Although the subgroup analysis uncovered some of the sources of variation, substantial heterogeneity persisted both between and within groups. Six families (Acipenseridae, Cichlidae, Mugilidae, Oplegnathidae, Salmonidae, Serranidae) showed substantial heterogeneity (I^2>75%), three families showed moderate heterogeneity (I^2 = 25-75%; Cyprinidae, Osphronemidae, Serrasalmidae) and only two families displayed modest heterogeneity (I^2 <25%; Bagridae, Clariidae). Variation among habitats, feeding guilds, and types of measurement were all substantial and not markedly different from the overall level of heterogeneity observed in the entire data set (I^2 = 96%). This suggests that other sources of variation are at play beyond those that could be accounted in the analysis.

Table 2.2. Sources of heterogeneity and subgroup analysis in the Spirulina dataset according to a random effects model.

Grouping	*k*	SMD	95%CI	*Q*	I^2
Family					
Acipenseridae	3	2.56	[-0.753; 5.884]	61.7	96.8%
Bagridae	5	-0.07	[-0.372; 0.232]	1.8	0.0%
Cichlidae*	38	1.20	[0.605; 1.802]	942.5	96.1%
Clariidae*	8	0.23	[0.100; 0.357]	5.7	0.0%
Cyprinidae	10	0.18	[-0.054; 0.412]	21.3	57.7%
Mugilidae*	10	5.11	[1.089; 9.121]	522.6	98.3%
Oplegnathidae	3	2.16	[-3.135; 7.459]	158.3	98.7%
Osphronemidae	4	0.19	[-0.430; 0.810]	10.6	71.7%
Salmonidae	13	0.15	[-0.859; 0.550]	194.5	93.8%
Serranidae	5	2.43	[-1.192; 6.055]	388.3	99.0%
Serrasalmidae	2	1.23	[-2.546; 5.001]	1.7	42.4%
Test for subgroup differences			*Q = 53.42, df = 10, P < 0.001*		
Habitat					
Freshwater*	82	0.705	[0.381; 1.022]	1531.2	94.7%
Marine*	19	3.557	[1.336; 5.777]	1092.0	94.8%
Test for subgroup differences			*Q = 7.11, df = 1, P = 0.008*		
Feeding					
Carnivores	26	0.680	[-0.094; 1.454]	936.2	97.3%
Omnivores*	28	1.862	[0.327; 3.396]	576.9	95.3%
Herbivores*	47	1.177	[0.667; 1.687]	1181.7	96.1%
Test for subgroup differences			*Q = 2.37, df = 2, P = 0.305*		
Measurement					
Individual data*	87	1.322	[0.776; 1.888]	2596.1	96.7%
Batch data	14	0.436	[-0.566; 1.437]	126.8	89.8%
Test for subgroup differences			*Q = 2.74, df = 1, P = 0.098*		

Groups that display a positive effect of Spirulina on standard growth rate are denoted by an asterisk. k = number of studies; SMD = standardized mean difference compared to controls; 95%CI = 95 % confidence interval around SMD; Q = Cochran's measure of heterogeneity; I^2 = percentage of variability unaccounted by sampling error.

2.3.2. Effects of *Schizochytrium* replacement on fillet omega-3 content

We found 14 quantitative studies on the effects of *Schizochytrium* replacement on omega-3 fillet content, representing k = 43 control-treatment comparisons. *Schizochytrium* studies were carried out in 10 species belonging to 9 different fish families. Study subjects ranged in size between 0.02 g and 850g (mean = 65.7g, SD = 183.3) and consisted of both marine and freshwater species in equal measure, although most results referred to carnivorous species (65%), such as Atlantic salmon (*Salmo salar* – 21% of studies) and red drum (*Sciaenops ocellatus* – 14% of studies). In most cases (79%), studies were carried out in triplicate tanks and involved an average of 132 individuals per tank (SD = 371), with feeding trials lasting between 21 and 133 days (mean = 64 days, SD = 25.8; **Table 2.3**). Replacement levels of *Schizochytrium* varied from 2% to 100% (mean = 42.6%, SD = 30.8).

Table 2.3. Results of feeding studies assessing the effects of Schizochytrium replacement on the omega-3 content of the fish fillet

Author	Study ID	SMD	Me	Se	Mc	Sc	Ne	Nc	`Species	Family	Water	Diet	Size (g)	Replac. %	Days	Tanks	Dens.	Data
Ortega.2016	s1	1.3714	33.30	4.67	25.30	4.67	3	3	*Epinephelus lanceolatus*	Serranidae	SW	C	45.9	100	84	3	6	batch
Ortega.2016	s1	1.4228	33.60	4.67	25.30	4.67	3	3	*Epinephelus lanceolatus*	Serranidae	SW	C	45.9	100	84	3	6	batch
Ortega.2016	s1	0.7371	29.60	4.67	25.30	4.67	3	3	*Epinephelus lanceolatus*	Serranidae	SW	C	45.9	68	84	3	6	batch
Ganuza.2008	s2	-0.3179	30.90	21.48	38.60	26.33	48	48	*Sparus aurata*	Sparidae	SW	C	0.15	100	21	3	NA	indiv.
Li.2009	s3	0.2896	4.12	4.76	2.73	4.76	50	50	*Ictalurus punctatus*	Ictaluridae	FW	O	20.4	10	63	5	15	indiv.
Li.2009	s3	0.5563	5.40	4.76	2.73	4.76	50	50	*Ictalurus punctatus*	Ictaluridae	FW	O	20.4	25	63	5	15	indiv.
Li.2009	s3	0.5229	5.24	4.76	2.73	4.76	50	50	*Ictalurus punctatus*	Ictaluridae	FW	O	20.4	40	63	5	15	indiv.
Li.2009	s3	0.7625	6.39	4.76	2.73	4.76	50	50	*Ictalurus punctatus*	Ictaluridae	FW	O	20.4	50	63	5	15	indiv.
Velazquez.2018	s4	-1.9846	15.47	0.05	16.46	0.67	9	9	*Sciaenops ocellatus*	Sciaenidae	SW	C	2.3	10	42	3	20	indiv.
Velazquez.2018	s4	-3.8690	14.53	0.05	16.46	0.67	9	9	*Sciaenops ocellatus*	Sciaenidae	SW	C	2.3	20	42	3	20	indiv.
Velazquez.2018	s4	-3.9550	14.09	0.45	16.46	0.67	9	9	*Sciaenops ocellatus*	Sciaenidae	SW	C	2.3	30	42	3	20	indiv.
Velazquez.2018	s4	3.1414	18.72	0.70	16.46	0.67	9	9	*Sciaenops ocellatus*	Sciaenidae	SW	C	2.3	40	42	3	20	indiv.
Velazquez.2018	s4	-1.1499	14.74	1.90	16.46	0.67	9	9	*Sciaenops ocellatus*	Sciaenidae	SW	C	2.3	50	42	3	20	indiv.
Velazquez.2018	s4	11.2993	22.22	0.15	16.46	0.67	9	9	*Sciaenops ocellatus*	Sciaenidae	SW	C	2.3	100	42	3	20	indiv.
Velazquez.2019	s5	-1.9048	15.50	0.10	16.50	0.70	9	9	*M crhysops ×M saxatilis*	Moronidae	FW	C	10.6	10	42	3	12	indiv.
Velazquez.2019	s5	-3.8095	14.50	0.10	16.50	0.70	9	9	*M crhysops ×M saxatilis*	Moronidae	FW	C	10.6	20	42	3	12	indiv.
Velazquez.2019	s5	-3.7577	14.10	0.50	16.50	0.70	9	9	*M crhysops ×M saxatilis*	Moronidae	FW	C	10.6	30	42	3	12	indiv.
Velazquez.2019	s5	2.9932	18.70	0.70	16.50	0.70	9	9	*M crhysops ×M saxatilis*	Moronidae	FW	C	10.6	40	42	3	12	indiv.
Velazquez.2019	s5	-1.1973	14.70	1.90	16.50	0.70	9	9	*M crhysops ×M saxatilis*	Moronidae	FW	C	10.6	50	42	3	12	indiv.
Sprague.2015	s6	-0.5111	17.70	3.40	20.40	6.00	6	6	*Salmo salar*	Salmonidae	SW	C	850	7.7	133	3	130	indiv.
Sprague.2015	s6	-0.2632	18.90	4.40	20.40	6.00	6	6	*Salmo salar*	Salmonidae	SW	C	850	21.1	133	3	130	indiv.
Miller.2007 FOc	s7	-2.3290	19.90	3.39	28.20	3.39	9	9	*Salmo salar*	Salmonidae	FW	C	40.0	20	63	3	24	indiv.
Miller.2007 FOc	s7	1.7392	33.10	1.70	28.20	3.39	9	9	*Salmo salar*	Salmonidae	FW	C	40.0	100	63	3	24	indiv.
Miller.2007 POc	s7	0.1969	19.90	3.39	18.90	5.94	9	9	*Salmo salar*	Salmonidae	FW	C	40.0	20	63	3	24	indiv.
Miller.2007 POc	s7	3.0961	33.10	1.70	18.90	5.94	9	9	*Salmo salar*	Salmonidae	FW	C	40.0	100	63	3	24	indiv.
Luo.2018	s8	0.0038	14.50	73.97	14.10	126.39	60	60	*Acipenser baerii*	Acipenseridae	FW	C	0.02	20	30	3	800	batch
Luo.2018	s8	0.0584	22.80	167.06	14.10	126.39	60	60	*Acipenser baerii*	Acipenseridae	FW	C	0.02	26.6	30	3	800	batch
Luo.2018	s8	0.0544	24.00	222.41	14.10	126.39	60	60	*Acipenser baerii*	Acipenseridae	FW	C	0.02	33.3	30	3	800	batch
Hoestenberghe.2016 FOc	s9	5.4713	5.70	0.30	4.00	0.30	12	12	*Scortum barcoo*	Terapontidae	FW	O	9.98	27.3	70	3	50	indiv.
Hoestenberghe.2016 POc	s9	10.6038	5.70	0.30	2.90	0.20	12	12	*Scortum barcoo*	Terapontidae	FW	O	9.98	27.3	70	3	50	indiv.
dos Santos.2019	s10	1.9676	2.20	0.07	1.80	0.24	4	4	*Oreochromis niloticus*	Cichlidae	FW	H	1.33	10	NA	4	12	indiv.
dos Santos.2019	s10	8.0407	3.50	0.10	1.80	0.24	4	4	*Oreochromis niloticus*	Cichlidae	FW	H	1.33	20	NA	4	12	indiv.
dos Santos.2019	s10	10.6223	4.20	0.14	1.80	0.24	4	4	*Oreochromis niloticus*	Cichlidae	FW	H	1.33	30	NA	4	12	indiv.
dos Santos.2019	s10	15.5822	5.20	0.12	1.80	0.24	4	4	*Oreochromis niloticus*	Cichlidae	FW	H	1.33	40	NA	4	12	indiv.
Seong.2019	s11	-0.7671	12.00	0.80	13.40	2.40	20	20	*Pagrus major*	Sparidae	SW	C	8.80	63.6	84	2	20	batch

Kousoulaki.2015	s12	0.3614	20.87	4.09	19.35	4.09	15	15	*Salmo salar*	Salmonidae	SW	C	213	2	84	3	40	batch
Kousoulaki.2015	s12	0.7490	22.50	4.09	19.35	4.09	15	15	*Salmo salar*	Salmonidae	SW	C	213	13.4	84	3	40	batch
Kousoulaki.2015	s12	-0.2449	18.32	4.09	19.35	4.09	15	15	*Salmo salar*	Salmonidae	SW	C	213	33.3	84	3	40	batch
Eryalçn.2015	s13	-7.5500	26.75	0.04	29.45	0.48	9	9	*Sparus aurata*	Sparidae	SW	C	0.02	75	90	3	2100	indiv.
Sarker.2016	s14	0.0139	26.90	39.44	26.50	3.29	15	15	*Oreochromis niloticus*	Cichlidae	FW	H	1.52	25	84	3	40	indiv.
Sarker.2016	s14	0.0825	27.00	7.67	26.50	3.29	15	15	*Oreochromis niloticus*	Cichlidae	FW	H	1.52	50	84	3	40	indiv.
Sarker.2016	s14	-0.0609	24.70	40.53	26.50	3.29	15	15	*Oreochromis niloticus*	Cichlidae	FW	H	1.52	75	84	3	40	indiv.
Sarker.2016	s14	-0.0291	26.10	18.62	26.50	3.29	15	15	*Oreochromis niloticus*	Cichlidae	FW	H	1.52	100	84	3	40	indiv.

SMD = Standardized Mean Difference; Me = Mean omega-3 content of experimental group; Se = standard deviation of omega-3 content of experimental group; Mc = Mean omega-3 content of control group; Sc = standard deviation of omega-3 content of control group; Ne = sample size of experimental group; Nc = sample size of control group; FW = freshwater; SW = sea water; Diet = Carnivore (C), Herbivore (H), Omnivore (O); Size= Initial mass (g); Replac. % = % Schizochytrium replacement level of fish oil (FO) or plan oil (PO); Days = duration of trial (days); Tanks = Number of replicate tanks; Dens. = Tank density (No. fish/tank); Data = type of measurement (individual measurements or batch measurement).

Effect sizes

Standardized mean differences (SMD), corrected for small sample sizes, varied between -7.6 and 15.6 and resulted in a pooled SMD of 0.621 (95%CI = -0.51-1.76) which is not significantly different from zero (t = 1.11, P = 0.274), and indicates that *Schizochytrium* inclusion in the diet does not have a positive overall effect on omega-3 fillet content (**Figure 2.6**). However, as with results from *Spirulina*, heterogeneity between studies was very high (Q = 37.7, df = 42, P < 0.001; I^2 = 88.9 τ^2 = 13.67) and the prediction interval was very wide (95% CI = -6.93; 8.18), indicating that both negative and positive effects on omega-3 fillet content are possible. Over 60% of control-treatment comparisons (23/43) were statistically significant, involving 6 of the 14 independent studies (43%). The average replacement of fish and plant oil with *Schizochytrium* that yielded an improvement in omega-3 fillet content was 16.2% (SD = 21.1), but positive effects were reported with *Schizochytrium* replacement as low as 10% in Nile tilapia (dos Santos, Schorer et al. 2019).

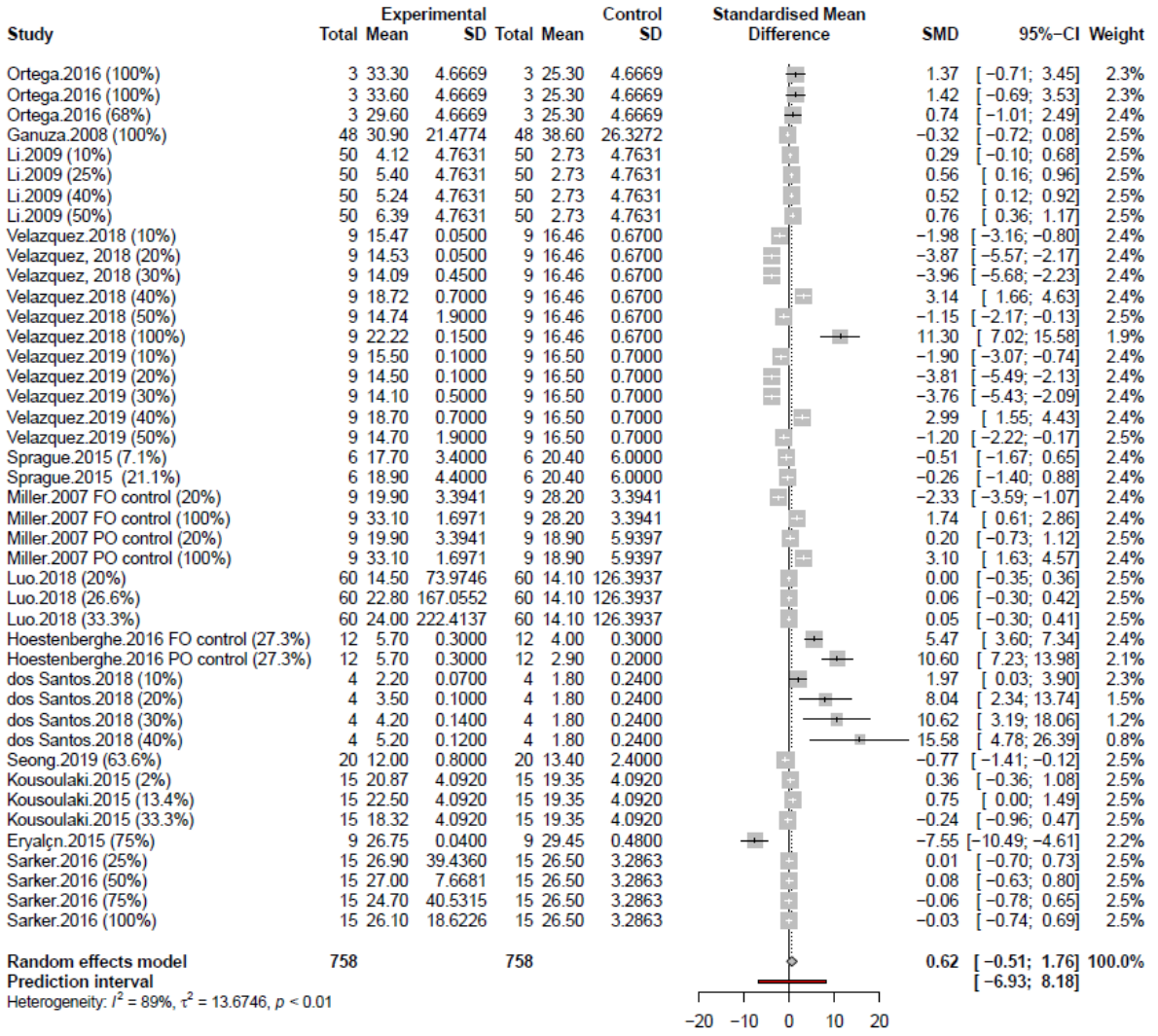


Figure 2.6. Forest plot summarizing the effect of Schizochytrium replacement on the omega-3 fillet total mean content of farmed finfish. Each trial (n = 43) is represented by a square whose size is proportional to its relative weight, its width represents the 95% CI, the horizontal line the 99% CI, and the center the Standardized Mean Difference (SMD) corrected for small sample size (Hedge's g). The grey diamond at the bottom represents the overall effect extending over the 95% CI. The solid vertical line denotes the zero effect, and the dotted vertical line the SMD under a random effects model.

Dose-effects

The level of *Schizochytrium* replacement was not a significant predictor of omega-3 fillet content (t = 1.574, df = 32, P = 0.125; **Figure 2.7**), but some differences were found among families. The family Terapontidae (*Scortum barcoo*, the Jade perch) showed a positive effect (t = 2.60, df = 32, P = 0.014), although this is based on only two points from a single study (Van Hoestenberghe, Fransman et al. 2016) and the amount of residual heterogeneity was high (Q_E = 222.53, df = 33, P < 0.001). Initial size was not a significant predictor of omega-3 fillet content (t = 0.102, df = 32, P = 0.919) and the best model only accounted for 20.8% of the observed heterogeneity ($F_{9,33}$ = 2.252, P = 0.043), driven by family effects.

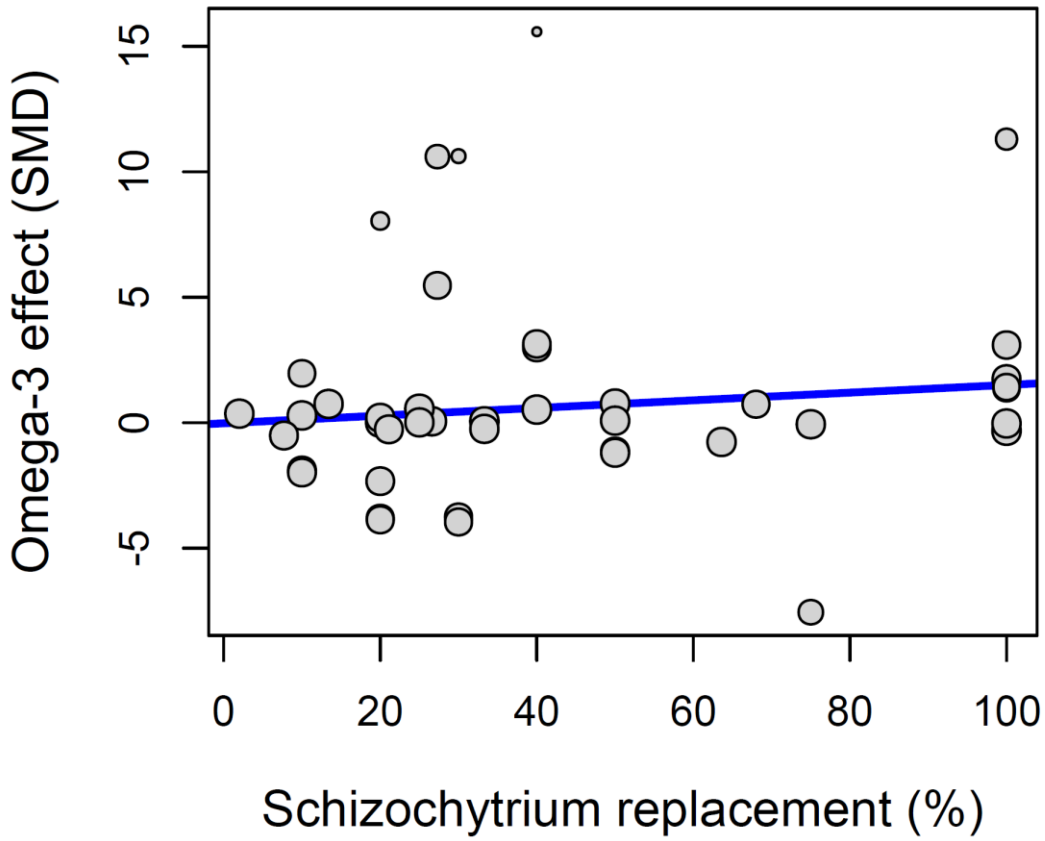


Figure 2.7. Bubble plot showing the estimated regression slope of the meta-regression on the effect of Schizochytrium replacement of total lipid (%) on the Standardized Mean Difference in the omega-3 fillet content. The size of the points is proportional to the weight of each study

Validity of results

As with *Spirulina*, a funnel plot of the *Schizochytrium* SMDs against their standard errors produced an asymmetric pattern (**Figure 2.8**) that might indicate the existence of publication bias. However the results of an Egger's test of funnel plot asymmetry was not significant (t_{41} = 0.55, P = 0.583; bias coefficient = 0.44, SE = 0.80), suggesting the is no evidence of publication bias.

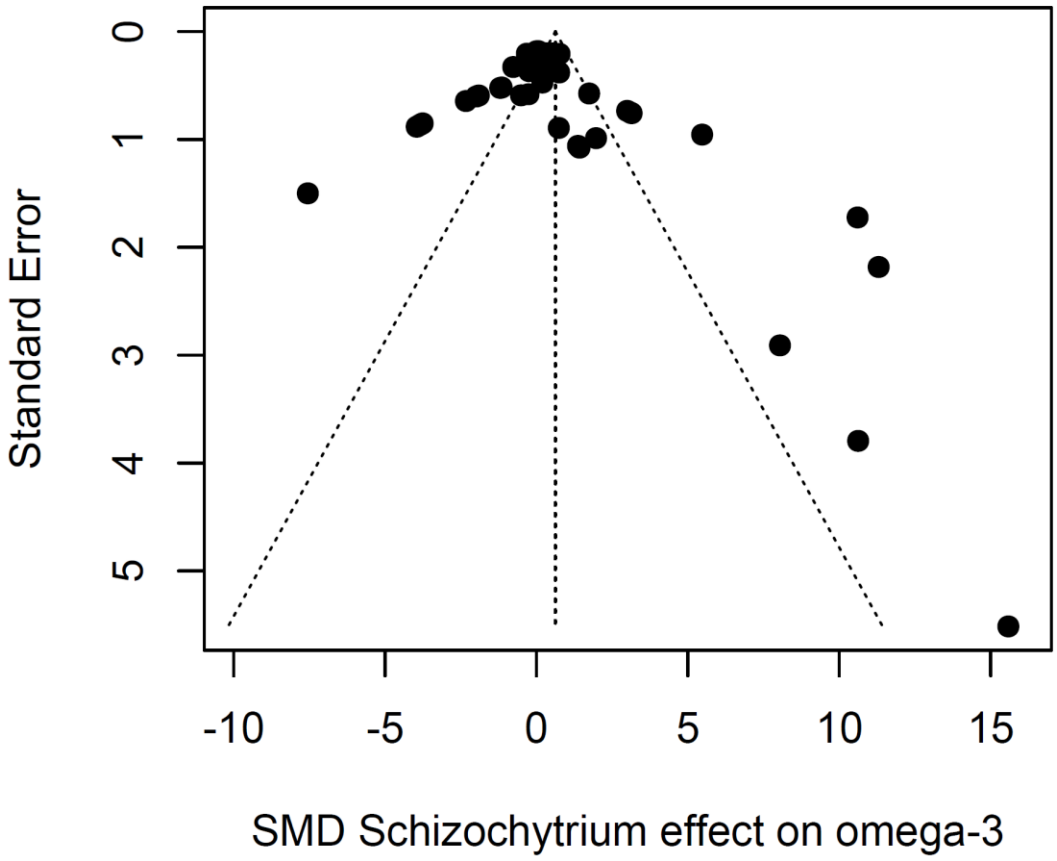


Figure 2.8. Funnel plot showing the relationship between the SMD and the standard error (inverted scale) for the effects of Schizochytrium replacement on omega-3 fillet content. Each point represents a treatment-control comparison, and the dotted vertical line denotes the global SMD under a random effects model. An asymmetric distribution of points outside the funnel might be indicative of publication bias.

Results from the *p*-curve test indicated that the distribution of significant results was significantly right skewed (*P* binomial < 0.001, full curve *P* < 0.001; half curve *P* < 0.001), which were confirmed by the flatness test (*P* binomial > 0.999, full curve *P* > 0.999; half curve *P* > 0.999). The evidential value indicates that the observed results are robust and unlikely to have been affected by publication bias.

Inspection of Baujat diagnostic plots detected one overly influential result like the ones of Li 2009 (**Figure 2.9**), while formal outlier analysis detected 14 extreme results. Reanalysis of the data without the overly influential point resulted in a pooled SMD of 0.63 (95%CI = -0.54; 1.80) which is not significantly different from zero (t = 1.09, P = 0.284). Removal of the 14 potential outliers resulted in a pooled SMD of 0.410 (95%CI = 0.005-0.815) which is marginally significantly different from zero (t = 2.08, P = 0.0473).

Taken together the results indicate that although there is no convincing evidence of a positive effect of Schizochytrium, it inclusion does not cause a loss of omega-3 content in the fish fillet. Heterogeneity, however, is substantial even when outliers are removed (I2 = 74.4%, Q = 109.2, df = 28, P < 0.001).

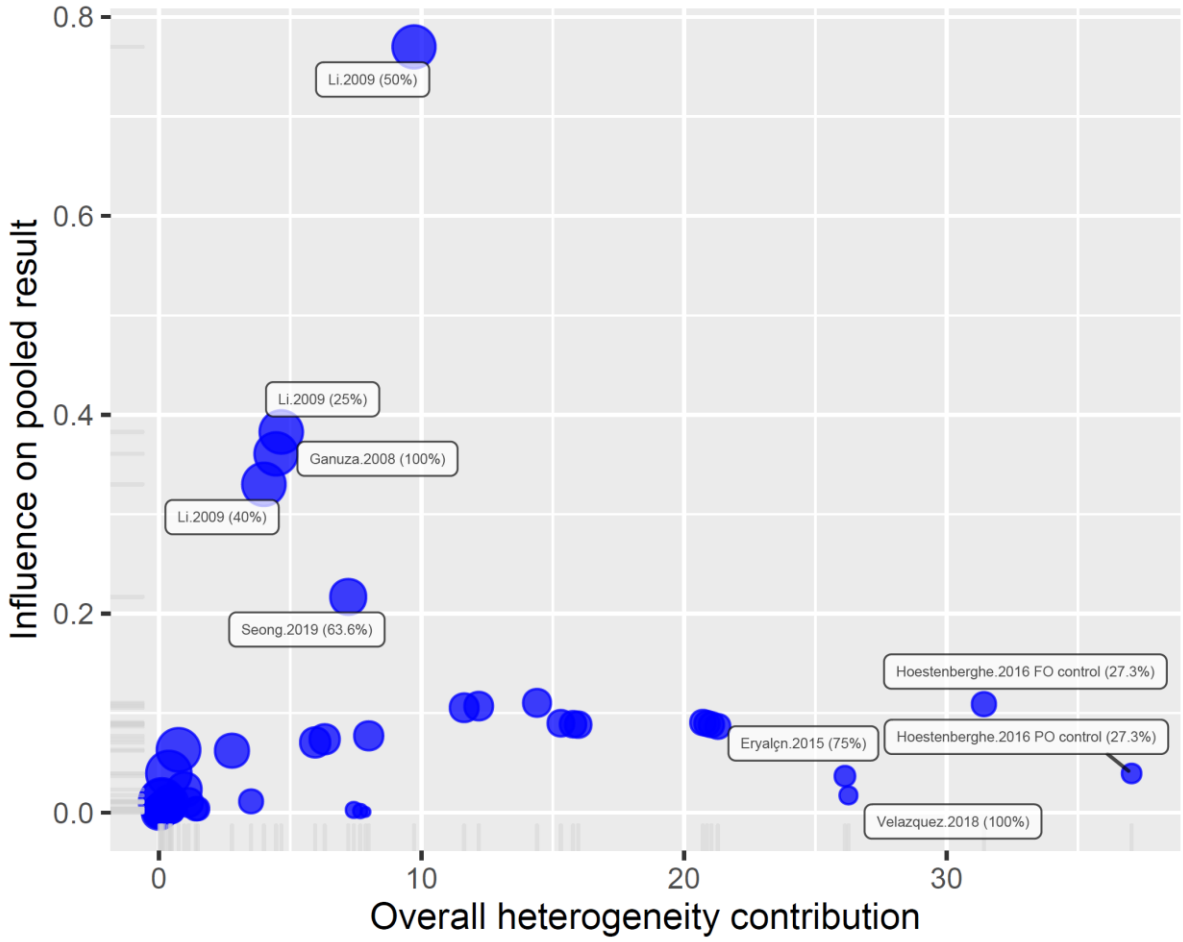


Figure 2.9. Baujat bubble plot used to identify potential outliers in the Schizochytrium data set, showing the contribution of each study to the overall heterogeneity and its influence under a random effects model. The percentage near the name of the author indicate the microalgal replacement level The size of each point is proportional to its relative weight in the meta-analysis. One trial in the upper left corner was overly influential which merited further scrutiny.

Subgroup analysis

Significant differences were found in *Schizochytrium* effects with respect to fish family (Q = 61.70, df = 8, P < 0.001), but not with respect to habitat (Q = 1.59, df = 1, P = 0.208), feeding guild (Q = 5.96, df = 2, P = 0..051) or type of measurements (Q = 0.75, df = 1, P = 0.387; **Table 2.4**). Of the 9 fish families examined, two families showed a statistically significant effect of *Schizochytrium* on omega-3 content (Ictaluridare SMD = 0.530; Serranidae SMD = 1.123) but the sample size is very small, the benefits modest and the uncertainty high.

Table 2.4. Sources of heterogeneity and subgroup analysis in the Schizochytrium dataset according to a random effects model.

Grouping	*k*	SMD	95%CI	*Q*	I^2
Family					
Acipenseridae	3	0.039	[-0.037; 0.114]	0.06	0.0%
Cichlidae	8	3.214	[-1.190; 7.618]	26.93	74.0%
Ictaluridare*	4	0.530	[0.222; 0.838]	2.70	0.0%
Moronidae	5	-1.513	[-4.944; 1.919]	55.02	92.3%
Salmonidae	9	0.292	[-0.828; 1.411]	43.22	81.5%
Sciaenidae	6	0.369	[-5.576; 6.313]	86.47	94.2%
Serranidae*	3	1.123	[0.132; 2.113]	0.32	0.0%
Sparidae	3	-2.266	[-12.481; 7.150]	23.49	91.5%
Terapontidae	2	7.820	[-24.670; 40.309]	6.79	85.3%
Test for subgroup differences			*Q = 61.70, df = 8, P < 0.001*		
Habitat					
Freshwater	22	1.368	[-0.449; 3.184]	186.80	88.8%
Marine	21	-0.051	[-1.537; 1.434]	179.42	88.9%
Test for subgroup differences			*Q = 1.59, df = 1, P = 0.208*		
Feeding					
Carnivores	29	-0.305	[-1.429; 0.819]	250.02	88.8%
Omnivores	6	2.806	[-1.433; 7.045]	3.95	92.0%
Herbivores	8	3.214	[-1.189; 7.618]	5.04	74.0%
Test for subgroup differences			*Q = 5.96, df = 2, P = 0.051*		
Measurement					
Individual data	33	0.132	[-0.261; 0.525]	362.00	91.2%
Batch data	10	0.806	[-0.743; 2.355]	14.76	39.0%
Test for subgroup differences			*Q = 0.75, df = 1, P = 0.387*		

Groups that display a positive effect of Schizochytrium on omega-3 content in the fish fillet are denoted by an asterisk. k = number of studies; SMD = standardized mean difference compared to controls; 95%CI = 95 % confidence interval around SMD; Q = Cochran's measure of heterogeneity;. I^2 = percentage of variability unaccounted by sampling error.

2.5 Discussion

Microalgae offer a potential solution to the growing need for more sustainable alternatives to fishmeal and fish oils in aquafeeds, and for healthier, more nutritional substitutes to plant oils (Shah, Lutzu et al. 2018), but high production costs and wide variation in the purported benefits have so far hampered a greater uptake by industry (Miller, Nichols et al. 2011, Chauton, Reitan et al. 2015, Hua, Cobcroft et al. 2019). The potential of microalgae to serve as sustainable replacement of animal or plant based protein and oils in aquafeeds has been extensively reviewed in recent years (Iyer, Prasad et al. 2011, Milledge 2011, Priyadarshani and Rath 2012, Enzing, Ploeg et al. 2014, Roy and Pal 2015, Sirakov, Velichkova et al. 2015, Vigani, Parisi et al. 2015, Révész and Biró 2019, Alagawany, Taha et al. 2021, Ansari, Guldhe et al. 2021), but surprisingly there is no quantitative global assessment of their nutritional benefits. Without a statistical analysis, it is difficult to determine to what extent the nutritional benefits of microalgae can be extrapolated across species, or depend on inclusion levels. For example, some authors have reported negative impacts of *Spirulina* at high inclusion levels in some species, while others have found no such constraints (Alagawany, Taha et al. 2021). To address these issues, we conducted a rigorous meta-analysis on the nutritional benefits of incorporating two of the most important microalgae, *Spirulina* and *Schizochytrium*, into aquafeeds for use in fish farming, assessed the extent and sources of variation, and critically examined various potential sources of bias.

Benefits of *Spirulina* replacement on fish growth

The results of our meta-analysis show that partial replacement of fish meal with *Spirulina* can have a significant positive effect on fish growth, with benefits being apparent from very modest inclusion levels, <1% and even less (Kermani, Babaei et al. 2020). However, growth benefits are dose-dependent and higher inclusion levels of *Spirulina* results in better growth. We estimated that a 1% increase in *Spirulina* inclusion increases specific growth rate by 0.07% compared to controls, although variability among families was very high. Growth was improved in 71% of the 17 species examined, but the best results occurred among the Cichlidae (tilapias), Clariidae (airbreathing catfishes), and Mugilidae (mullets), species which are all herbivorous.

Negative results were also found, although these instances were rare. Loss of weight compared to controls following replacement with *Spirulina* was reported in 5% of studies (**Figure 2.10**) and involved three species: Nile tilapia at 2-2.7%% replacement (El-Ward, Eid et al. 2016,

Mahmoud, El-Lamie et al. 2018), mullet at 3.9% replacement (Rosas, Monserrat et al. 2019) and rainbow trout at 0.1-4% replacement (Güroy, Güroy et al. 2019, Kermani, Babaei et al. 2020). In most cases (95%), however, *Spirulina* either improved growth or had no negative effect compared to controls and replacements of up to 40-45% have been used without detrimental impacts in several species (Hussein, Dabrowski et al. 2013, Ribeiro, Leite et al. 2019, Rosas, Monserrat et al. 2019, Rosas, Poersch et al. 2019). *Spirulina* is currently used as a supplement in aquafeeds, rather than as a total replacement, in line with its use as a prebiotic in animal and human nutrition (Finamore, Palmery et al. 2017).

Benefits of *Schizochytrium* replacement on omega-3 fillet content

Ingestion of suitable n3 LC PUFA is essential for proper egg development and offspring survival (Tocher 2010) and *Schizochytrium* represents a sustainable and rich source of DHA for maturing fish (Ling, Guo et al. 2015). Moreover, given the importance of early life for subsequent development (Ellison, Uren Webster et al. 2020, Uren Webster, Rodriguez-Barreto et al. 2020), the essential fatty acids provided by *Schizochytrium* and other similar thraustochytrids can have long-term beneficial effects on fish health and growth, as seen in Siberian sturgeon, (Luo, Ai et al. 2019), Nile tilapia (Sarker, Gamble et al. 2016, Sarker, Kapuscinski et al. 2016, dos Santos, Schorer et al. 2019, de Souza, de Lima et al. 2020), red sea bream (Seong, Kitagima et al. 2020), channel catfish (Li, Robinson et al. 2009), and jade perch (Van Hoestenberghe, Fransman et al. 2016).

We did not find a positive global increase in omega-3 in the fish fillet compared to controls, but the mean SMD was not statistically different from zero, indicating that replacement of fish oil or plant oil with *Schizochytrium* oil is possible without a significant loss of omega-3 content. Indeed, positive or neutral (i.e. zero-effect) results were reported in 74% of the trials (**Figure 2.10**). The 26% of cases where the omega-3 content of the fish fillet deteriorated after *Schizochytrium* inclusion refer to studies involving five species: red drum (Perez-Velazquez, Gatlin III et al. 2018), hybrid striped bass (Perez-Velazquez, Gatlin III et al. 2019), Atlantic salmon (Miller, Nichols et al. 2007), red seabream (Seong, Matsutani et al. 2019), and gilt-head bream (Eryalçin and Ildiz 2015). The absence of a dose effect means that 100% substitution of animal or plant oils with *Schizochytrium* oil is possible and should not decrease the nutritional value of the fish fillet, as demonstrated for Nile tilapia (Sarker, Kapuscinski et al. 2016), although variability is very high and the prediction interval wide, and this introduces considerable uncertainty on the expected results.

Heterogeneity between studies and sources of variation

We found substantial heterogeneity in the results of fish feeding studies using *Spirulina* (I^2 = 96%) and *Schizochytrium* (I^2 = 89%). The relative frequency of different outcomes (positive, neutral, and negative results) differs significantly between *Spirulina* and *Schizochytrium* studies ($\chi 2$ = 11.197, *df* = 2, *P* = 0.004; **Figure 2.10**). Non-negative results (i.e. positive plus neutral) were more common for *Spirulina* effects on growth (94%) than for *Schizochytrium* effects on omega-3 fillet content (74%), confirming the results of the two meta-analyses, which yielded a significant non-zero global effect for *Spirulina* (95%CI SMD = 0.71-1.70) but included zero in the case of *Schizochytrium* (95%CI SMD = -0.51-1.76). Highly variable outcomes are common in microalgal studies. For example, Ahmad, Shariff et al. (2020) reported 36% significant improvements in 11 studies that examined changes in growth or fillet quality following inclusion of *Chlorella vulgaris* in aquafeeds, 36% with no discernible benefit, and 27% negative effects, which were apparently exacerbated at high inclusion levels.

High heterogeneity in meta-analysis is problematic because it makes it difficult to generalize across contexts (Borenstein 2019, Borenstein, Hedges et al. 2021). Heterogeneity can be caused by clinical (or structural) differences between subjects and how they respond to treatments, but also by methodological differences in study design, and by statistical variation in intervention effects (Fletcher 2007, Deeks, J.P.T. et al. 2011). We dealt with high heterogeneity by performing meta-regression and by conducting subgroup analysis (Ioannidis, Patsopoulos et al. 2008). We found that family effects were the main source of heterogeneity (Q = 61.70), but this only explained a small part of the observed variation (~24-27%). Most of the variation could not be explained by differences in the way different fish families responded to microalgae replacement, or by variation in microalgae inclusion levels, differences in fish size, habitat, feeding guild or the way the data were recorded.

It is likely that other, unaccounted, biotic and abiotic sources of variation contributed to the high observed level of heterogeneity (Kestemont, Jourdan et al. 2003). For example, fish growth can vary enormously depending on sex and stocking density (Sánchez, Ambrosio et al. 2010), water temperature (Handeland, Imsland et al. 2008), photoperiod (Handeland and Stefansson 2001), light intensity (Strand, Alanärä et al. 2007), tank size (Espmark, Kolarevic et al. 2017), tank colour (Strand, Alanärä et al. 2007), social status (Huntingford and Garcia de Leaniz 1997), trial duration, seasonality and feeding rates (Du, Liu et al. 2006). These are likely to differ between studies but are seldom reported. Likewise, substantial variation has also been reported in the fatty acid composition of fish fed identical diets under communal rearing conditions (Schlechtriem, Bron et al. 2007), suggesting that individual differences in deposition

of omega-3 can be substantial. The nutritional value of micro-algae also differs between strains and producers (Muys, Sui et al. 2019), depending on culture conditions (Richmond 2008), geographic location (Winwood 2013), and post-harvest treatment (Becker 2007, Dewi, Amalia et al. 2016, Batista, Pintado et al. 2020) adding additional sources of unaccounted variation.

Microalgae can provide substantial benefits to aquaculture nutrition but poorly planned and/or executed studies hamper progress, waste time and money, and nullify the results of more robust trials. We urge authors to adhere to accepted guidelines for reporting results of fish studies, including mean effects, sample sizes and measures of variability (Brattelid and Smith 2000), as well as ethical considerations (McGrath, Drummond et al. 2010). It might also be beneficial to exclude dubious studies from future meta-analysis, or to weigh studies by some measure of reliability (Borenstein, Hedges et al. 2021, Harrer, Cuijpers et al. 2021).

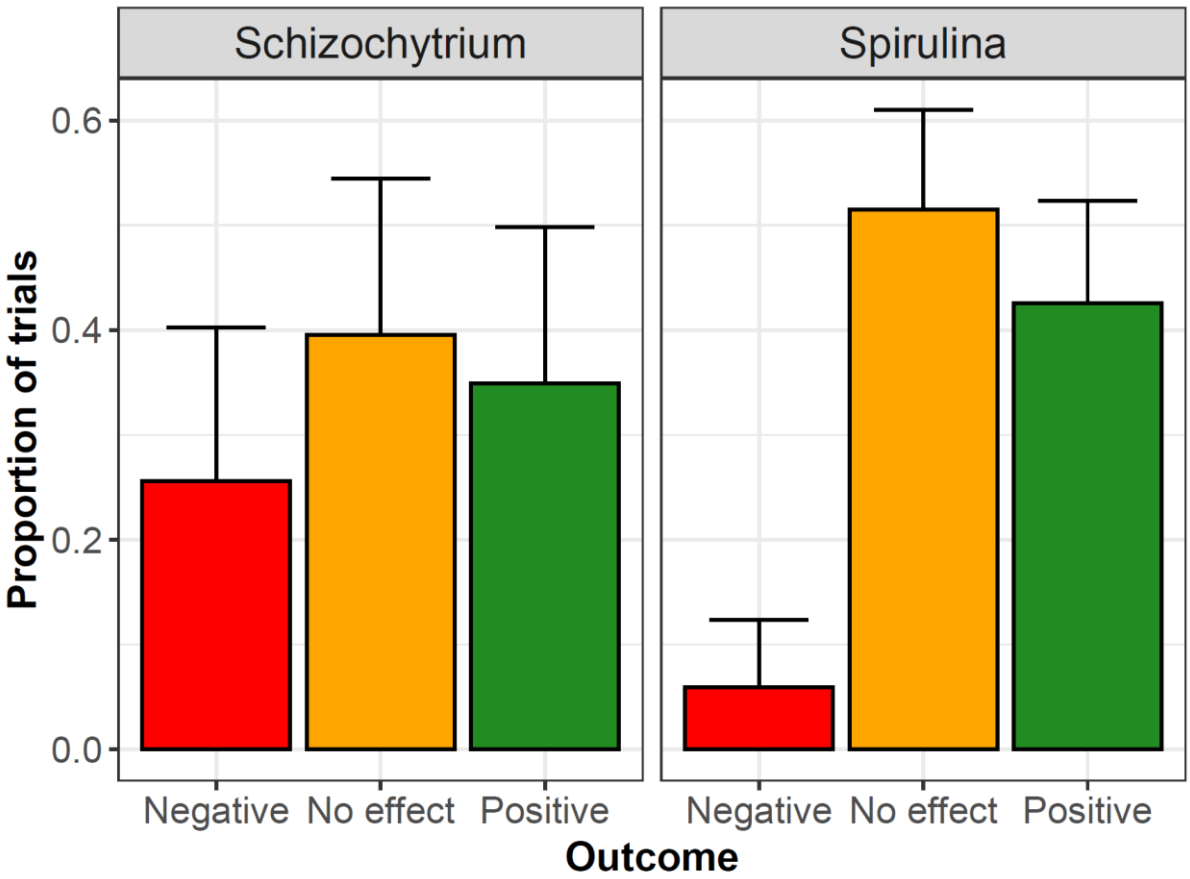


Figure 2.10. Breakdown of study outcomes (SMDs) under a random effects model for the Schizochytrium and Spirulina meta-analyses, showing the proportion of statistically significant negative effects, no effect, and positive effects along with the 95% binomial CI. The distribution of non-negative outcomes is significantly better for Spirulina than for Schizochytrium studies ($\chi 2 = 11.197$, df = 2, P = 0.004).

Publication bias

We found no clear evidence of systematic publication bias. Plotting effect sizes against standard error of the estimates resulted in asymmetric funnel plots for both *Spirulina* and *Schizochytrium* which can be indicative of publication bias (Sterne and Egger 2005). However, asymmetry could not be confirmed by the more explicit Egger's tests (Egger, Smith et al. 1997) in the case of *Schizochytrium* and the results of p-curve analysis (Simonsohn, Nelson et al. 2014) indicated that there was sufficient evidential value for both micro-algae, suggesting there was an underlying true effect. Publication bias could have been masked by high study heterogeneity which may have diminished the power of the p-curve method (van Aert, Wicherts et al. 2016), but our sensitivity analysis indicates that the pooled effect sizes calculated for *Spirulina* and *Schizochytrium* were robust to the exclusion of outliers and overly influential points.

Wider benefits of using microalgae in aquafeeds

There are over 40 different species of micro-algae used in fish farming, but these
are mostly used to feed rotifers and copepods to wean fish larvae, or are administered live directly to fish reared in 'green waters' (Lavens and Sorgeloos 1996, Oostlander, van Houcke et al. 2020). Only ~19 microalgae are used as part of formulated aquafeeds (Shah, Lutzu et al. 2018, Nagappan, Das et al. 2021), production being dominated by freshwater species such as *Spirulina* which is the dominant species with 41% of the global market due its ease of culture, nutrient profile, and high yield (Future Market Insights 2021).

Although live microalgae are a staple feed in many fish hatcheries (Brown and Blackburn 2013), ingestion rates are difficult to control in 'green waters' and their use is typically restricted to larval stages. In contrast microalgae-based aquafeeds can be used at all stages of fish development, offering superior control over feeding, necessary for precision aquaculture (Føre, Frank et al. 2018). Also, unlike plant-based aquafeeds that are difficult to be accepted by carnivorous species (Kortner, Björkhem et al. 2014), microalgae incorporated into aquafeeds can be used to feed both carnivorous and herbivorous species (Ansari, Guldhe et al. 2021). Many microalgae have rigid cell walls which results in low digestibility (Niccolai, Zittelli et al. 2019), but new technical solutions are being developed to overcome this challenge (Agboola, Teuling et al. 2019, Teuling, Wierenga et al. 2019, Ansari, Guldhe et al. 2021).

Not all species of microalgae are as rich in n3 LC PUFA as *Schizochytrium* (Sarker, Kapuscinski et al. 2016), or have the high protein content of *Spirulina* (~63-65%) to replace fish meal (Becker 2007) but combining different microalgae can overcome this limitation. For example, *Schizochytrium* represents a good source of DHA for maturing fish, but is poor in EPA (Ling, Guo et al. 2015), but by combining it with oil from *Nannochloropsis* which is rich in EPA (Chua and Schenk 2017) an appropriate balance of omega-3 fatty acids can be ensured, necessary for the production of high quality gametes (Holt 2011). Likewise, while *Schizochytrium* oil possess a nutritional profile similar to fish oil (Winwood 2013, Sarker, Gamble et al. 2016, Sarker, Kapuscinski et al. 2016), *Spirulina* lacks essential amino-acids compared to fish meal, which can potentially reduce growth at high inclusion levels for some species (Roy and Pal 2015, Raji, Jimoh et al. 2020). Thus, different combinations of microalgae may be required to meet the nutritional needs of different fish species (Sarker, Kapuscinski et al. 2020). Yet, few studies have compared the benefits of combining different proportions of microalgae and this is an area where more research is clearly needed.

One advantage of microalgae over plant-based aquafeeds is that their benefits are not limited to enhanced growth or nutritional value, but can also extend to fish health as well (Shah, Lutzu et al. 2018, Camacho, Macedo et al. 2019). Microalgae are increasingly being considered for their therapeutic properties, in addition to their nutritional aspects (Sushma and Sharma 2021). For example, *Spirulina* and *Chlorella* can boost the immune system of fish (Ahmad, Shariff et al. 2020, Al-Deriny, Dawood et al. 2020), and *Spirulina* may also have anti-viral properties (Chen, Chang et al. 2016). Incorporation of *Spirulina* in the fish diet was reported to enhance hepatic antioxidant function and disease resistance in coral trout, *Plectropomus leopardus* (Yu, Wen et al. 2018), great sturgeon, *Huso huso* (Adel, Yeganeh et al. 2016), Nile tilapia (Abdel-Latif and Khalil 2014, El-Murr, Abd Elhakim et al. 2014, Mahmoud, El-Lamie et al. 2018), African catfish (Raji, Junaid et al. 2019), mullet (Rosas, Bessonart et al. 2019), as well as in several cyprinids (Cao, Zhang et al. 2018, Viswanathan and Arockiaraj 2019) and salmonids (Kermani, Babaei et al. 2020, Meshkat Roohani, Fallahi Kapoorchali et al. 2020). Inclusion of *Spirulina* at 8-10% was also found to increase fecundity in three-spot gourami (Khanzadeh, Fereidouni et al. 2016).

Maximizing the value of feeding studies using microalgae

In common with other meta-analysis in aquaculture (Fagnon, Thorin et al. 2020), we found it difficult to extract the necessary information from fish feeding trials to ascertain effect sizes. A surprisingly large number of studies do not provide enough information to replicate the work,

or to ascertain the experimental validity of the results. Results of feeding trials using microalgae have increasingly been published with little or no rigorous peer reviewing (or where peer-reviewing takes place, this is measured in days, rather than in months). Of 1,474 studies we screened, only 3% were eligible for analysis.

In the studies reviewed 14% of trials involved batch measurements in the case of *Spirulina* and 23% in the case of *Schizochytrium*, and this may have also introduced some biases. Batch measurements are not recommended as they can mask important sources of variation, reduce sample size (and thus statistical power) and may result in inflated effect sizes, which can be misleading. Researchers should also be clear about the unit of replication, take into account the nested nature of the data and the statistical power to detect differences, particularly in growth studies (Ling 2007, Thorarensen, Kubiriza et al. 2015). For example, there is little benefit in using triplicate tanks if tank effects are ignored and data are pooled. Fish can now be individually marked since a young age and small size (Faggion, Sanchez et al. 2020), which is essential for precision fish farming (Føre, Frank et al. 2018), and tank effects can be accounted for using linear mixed effects models (Thorarensen, Kubiriza et al. 2015).

All results we reviewed were based on feeding trials typically carried out in comparatively small tanks or enclosures under relatively low densities, which are unlikely to be representative of commercial conditions. Given the high heterogeneity found in effect sizes, there is some uncertainty about the wider applicability of the reported results. There is clearly a need to examine the performance of algae-enriched aquafeeds under commercially relevant conditions that extend over longer time periods than the average 60-day feeding trial to ascertain the validity and potential limitations of upscaling (Cottrell, Blanchard et al. 2020).

2.6 Outlook and Conclusions

Our meta-analyses examined the nutritional benefits of only two species of microalgae, *Spirulina* and *Schizochytrium*, but these represent the main ones, and also the ones where there was enough quantitative data to conduct a statistical analysis. The results indicate that inclusion of *Spirulina* in the fish diet improves standard growth rate overall, while replacement of fish or plant oil with *Schizochytrium* oil is possible without loss of omega-3 content in the fish fillet in 71% of the cases involving 91% of the species examined. However, the results are very heterogenous and depend not only on fish species, but in the case of *Spirulina* also on

inclusion level, as well as on unaccounted sources of variation likely related to differences in the way feeding trials were conducted. We found no clear evidence of publication bias, and the results were generally robust to exclusion of extreme values.

The Aquaculture industry will be worth $50.6 billion by 2026 (360iResearch 2021), the main cost of which will continue to be the cost of aquafeeds (Peñalosa Martinell, Cashion et al. 2020, Lucintel 2021). The use microalgae in aquafeeds is still more expensive than using fishmeal, fish oils or plant crops (Hua, Cobcroft et al. 2019) but the price of fish meal has increased more than 200% over the last two decades (Indexmundi 2021). As microalgae production becomes cheaper and more efficient (Torres-Tiji, Fields et al. 2020), microalgal-based aquafeeds will become more competitive (Sarker, Kapuscinski et al. 2020). Production of *Spirulina* is expected to be worth $4.6 billion by 2027 (Global Industry Analysts Inc. 2021), mostly driven by the nutraceutical, food and beverage segment, but also by aquaculture (Meticulous Market Research 2021). To speed the transition towards more sustainable, zero-catch aquafeeds, rigorous comparative analyses of novel microalgal diets are needed, but results need to be directly relevant to the feed manufacturers (Turchini, Trushenski et al. 2019). To achieve this, we recommend that feeding trials using microalgae are conducted under commercially relevant conditions that consider the challenges of upscaling, and that greater care is taken to report the raw data and to include full rearing details, along with units of replication, mean effects, sample sizes and measures of variability.

CHAPTER 3 – EFFECTS OF MICRO-ALGAE OIL REPLACEMENT ON GROWTH, OMEGA-3 DEPOSITION AND GUT MICROBIOME COMPOSITION IN NILE TILAPIA (*Oreochromis niloticus*)

3.1 Abstract

Microalgae offer a sustainable source of omega-3 fatty acids that can replace fish oil in aquafeeds, but the nutritional benefits are not always clear, particularly when microalgae are used as complete oil replacements in starter feeds. We compared the survival, growth, omega-3 deposition and composition of the gut microbiota of Nile tilapia fed with aquafeeds that differed in dietary oil, from plant, fish and microalgae (*Schizochytrium*) origins. Survival was not affected by diet, but fish fed a diet where the entire oil component (5%) was replaced by microalgae oil grew twice as fast as fish fed plant oil or a mixture of plant and fish oil. Dietary omega-3 content was strongly correlated with omega-3 deposition in the fish fillet. Complete replacement of fish oil by plant oil caused a significant increase in the abundance of Aeromonadaceae which is often associated with an inflammatory response in the fish gut. In contrast, when fish oil was replaced by microalgae oil an increase in Peptostreptococcaceae and Mycobacteriaceae was observed. Our study indicates that microalgae oil replacement can be used to improve growth and make Nile tilapia more nutritious by increasing its omega-3 content without any of the detrimental effects on the gut microbiome typically associated with plant oil replacement.

3.2 Introduction

Nile tilapia (*Oreochromis niloticus*) is the second most widely farmed fish worldwide (Prabu, Rajagopalsamy et al. 2019) and the main source of fish food for millions of people, particularly in developing countries (Fitzsimmons, Martinez-Garcia et al. 2011). The reasons of its success lie in its fast growth, hardiness, and herbivorous diet, which makes tilapia feeds relatively cheap (Köprücü and Özdemir 2005, da Silva Dias, Pereira et al. 2020). However, although farmed tilapia can help alleviate food insecurity and reduce the risk of malnutrition (Bene and Heck 2005, Gupta 2006), they are typically fed a high percentage of plant ingredients (El-Sayed 1999), which results in low omega-3 content (Osibona, Kusemiju et al. 2009) and diminishes their nutritional value (Karapanagiotidis, Bell et al. 2006), impacting those countries where access to healthy food is most needed. Improving the nutritional value of Nile tilapia is, therefore, a priority for many developing countries (Hasselberg, Aakre et al. 2020, Bhujel and Suharman 2021).

An adequate dietary intake of eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) long-chain polyunsaturated fatty acids (omega-3 for short), is essential for the regulation of many metabolic pathways (Hussein, Attia et al. 2019), the adequate functioning of the retina and growth of neural tissue (Politi, Rotstein et al. 2001, Lauritzen, Brambilla et al. 2016), the development of cognition (Liu et al., 2020 (Sinn, Milte et al. 2012, Zhou, Ding et al. 2018, Liu, Wang et al. 2020), and the control of the inflammatory response (Alfaddagh, Elajami et al. 2018). Omega-3 present in aquafeeds are deposited in the fish fillet and are then transferred to human consumers (Sissener 2018), offering possibilities for improving the diet of millions of people through fish farming. To make tilapia more nutritious, aquafeeds can be enriched with fish oil (Kris-Etherton, Harris et al. 2002), but this is costly (Rana, Siriwardena et al. 2009) and increasingly unsustainable (Chuenpagdee, Degnbol et al. 2005), as fish oil is obtained from depleted wild stocks (FAO 2020). Fish oil is often replaced by plant oils, but these do not have the same omega-3 content or composition of fish oil (Pickova and Mørkøre 2007) and this has also been associated with some adverse health effects on fish health including intestinal inflammation (Dawood 2021).

Unicellular microalgae and cyanobacteria represent sustainable sources of omega-3 fatty acids for human and animal nutrition (Sarker, Kapuscinski et al. 2018, Shah, Lutzu et al. 2018, Sarker, Kapuscinski et al. 2020). The nutritional profile of some microalgae is comparable to that of fish oil without any of the sustainability drawbacks (Lum, Kim et al. 2013). An

alternative to the use of fish oil in aquafeeds, therefore, would be to use oil extracted from microalgae rich in omega-3, which is higher in omega 3 than plant oil (Shah, Lutzu et al. 2018). Microalgae could be used to fortify the diets of farmed fish and increase the nutritional value of fish food (Ryckebosch, Bruneel et al. 2014, Sarker, Kapuscinski et al. 2018).

Among the microalgae currently being cultivated, the marine heterotrophic *Schizochytrium* sp. is the only one whose oil is commercially available for incorporation into fish diets (Tocher, Betancor et al. 2019). *Schizochytrium* possess two important qualities that make it attractive for use in aquafeeds: its oil is rich in omega-3 - up to 40-45% Docosahexaenoic acid (DHA) and 10% Eicosapentaenoic acid (EPA) (Fedorova-Dahms, Marone et al. 2011) and, unlike other microalgae which require specific carbon sources to grow, *Schizochytrium* can thrive on agriculture by-products (Carr, 2017) and even wastewater from fish farming (Jung & Lovitt, 2010), which facilitates its culture. High production costs are the main factor limiting the uptake of microalgae in aquafeeds (Ansari, Guldhe et al. 2021), but recent advances in algal biotechnology have made microalgae production more cost-effective (Koyande, Chew et al. 2019).

Recent studies have shown that the omega-3 content of Nile tilapia can be enhanced by inclusion of *Schizochytrium* (Sarker, Kapuscinski et al. 2016, Sarker, Kapuscinski et al. 2018, dos Santos, Schorer et al. 2019, Sarker, Kapuscinski et al. 2020), but there are uncertainties regarding the optimal level of algal inclusion and the form of administration, as most studies have used whole microalgae rather than microalgae oil, which may contain anti-nutrients and can affect the digestibility of fatty acids and nutrient uptake (Teuling, Wierenga et al. 2019).

The amount of omega-3 in the diet can alter the composition of commensal bacteria in humans (Menni, Zierer et al. 2017) and mice (Davis, Hecht et al. 2017), but whether the same happens in fish is not known. Inclusion of microalgae in aquafeeds can deliver multiple benefits (Shah, Lutzu et al. 2018), but the effects on the fish gut microbiome have seldom been explored. Previous studies have shown that inclusion of the microalgae *Schizochytrium* in the diet does not impact on fish welfare (Emery, Norambuena et al. 2016), and could improve growth and the omega-3 content in the fish fillets (Watters, Rosner et al. 2013, Sarker, Gamble et al. 2016), but its effects on the gut microbiome are unknown. Gut microbiota possess strong connection with immunity (Ellis 2001) as well as producing sever enzymes which support fish digestion (Wu, Ren et al. 2015).

Here, we assessed the effects of varying the amount and sources of dietary oil (microalgae, fish and plant) on omega-3 deposition, growth, survival, and composition of the gut microbiome of Nile tilapia while keeping other dietary ingredients constant. We carried out study from first feeding until 94 days, as this is the time when the fish microbiome is most plastic and most likely to be affected by changes in the diet and by bacterial colonization history (Uren Webster, Rodriguez-Barreto et al. 2020). Both fish and microalgae oil are rich in omega-3 fatty acids, but microalgae oil has a higher DHA:EPA ratio than fish oil, which enabled us to assess the effects of different fatty acid profiles on tilapia weaning and subsequent performance.

3.3 Materials and Methods

Experimental design and sampling

We obtained three day old, mixed sex Nile tilapia (Silver strain) from Stirling University, originating from three half-sib families (1 male, 3 females). Fish were housed in 18 x 25L plastic tanks at the Centre for Sustainable Aquaculture Research (Swansea University, Swansea UK). Rearing conditions and water quality were maintained within optimal conditions for Nile tilapia (El-Sayed 2013): temperature 27–28°C, dissolved oxygen > 4.0mg/L, photoperiod 12D:12L.

Eighteen tanks, each containing 90 fish, were assigned at random to six experimental diets (see below) in triplicate. Fish were fed to satiation three times per day. Four fish per tank (12 per dietary group) were humanely sacrificed using an overdose of phenoxyethanol followed by destruction of the brain according to UK Home Office regulations and sampled at day 3 (average fish size at start 0.024gr), and then every ~3 weeks at days 21, 43, 63 and 94. Fish were sampled before they were fed for the day to avoid full guts, as the microbiota of the feed was not sequenced. The whole gut of the fish was dissected using aseptic techniques and preserved in RNA-later (QIAGEN N.V.) at -23 ºC. Total length and weight were recorded for growth analysis, and Fulton's condition factor (K) was calculated as a measure of body condition (Bolger and Connolly 1989).

Dietary formulation

Six experimental tilapia diets (**Table 3.1**) were formulated according to the nutritional requirements detailed in Royes and Chapman (2003). Diets varied only in terms of oil source and composition, the remaining ingredients being otherwise identical. A control (reference) diet included 50% salmon oil (as this was readily available in small quantities suitable for this experiment) and 50% soya oil to represent commercial diets, which usually contain a mix of fish and plant oils. The other five experimental diets were formulated to replace the control diet with increasing proportions (33%, 66%, 100%) of oil from the microalgae *Schizochytrium* sp. (Henry Lamotte Oils GmbH, Germany) or with 100% fish oil or 100% plant oil. Unlike some other studies, we used *Schizochytrium* oil instead of the whole microalgae to make it comparable with the other dietary oils.

Table 3.1. Formulation (g/1000g) of the six experimental diets for juvenile Nile tilapia. Pellet size at first feeding was 250nm, then increased to 400nm,600nm, 800nm every three weeks.

	CON	FISH	PLANT	ALGAE33	ALGAE66	ALGAE100
Maize	134	134	134	134	134	134
Wheat	200	200	200	200	200	200
Wheat Bran	80	80	80	80	80	80
Wheat gluten	125	125	125	125	125	125
Line Seed Meal	125	125	125	125	125	125
Fish meal	141	141	141	141	141	141
Soya bean meal	135	135	135	135	135	135
Fish oil	25	50	--	16.75	8.5	--
Soya oil	25	--	50	16.75	8.5	--
Schizochytrium oil	--	--	--	16.5	33	50
Vitamin and mineral premix	10	10	10	10	10	10

Fatty acid analysis

A fatty acid profile of the different diets was carried out by Sciantec Analytical (Cawood Scientific, UK). Total fat and omega-3 content (EPA+DHA) of Nile tilapia muscle at the end of the trial (day 94) was analysed by Campden BRI (UK). Total fat content in the fish muscle (g fat/100 g muscle) was determined using the Weibull-Stoldt method. Omega-3 content (EPA+DHA) was determined as Fatty Acid Methyl Esters (FAMEs) and is reported as percentage of omega-3 fatty acids in the muscle fat to make it more comparable to the FA results of the experimental diets. Three fish were pooled together for each diet due to their small size (around 3 to 5 grams per fish).

16sRNA library preparation and sequencing

DNA was extracted from 72 gut samples collected on days 21 and 94, using the DNeasy PowerSoil PowerLyser kit (Qiagen), according to the manufacturer's instructions. 16S rRNA libraries were prepared using the Earth Microbiome primers 515F and 806R (Walters, Hyde et al. 2016) to target the V4 hypervariable region, based on the Illumina Metagenomic Sequencing Library Preparation protocol (Illumina) using Platinum Hot Start Taq Polymerase (Invitrogen). All libraries were purified using AMPure XP magnetic beads (Beckman Coulter), indexed using Nextera XT indices (Illumina), multiplexed in equimolar concentrations and sequenced using an Illumina MiSeq platform (2x 300 bp). Two extraction blanks were prepared and sequenced alongside the gut samples. The demultiplexed 16s sequences were denoised and clustered with DADA2 (Callahan, McMurdie et al. 2016) within QIIME 2 2020.2 (Bolyen, Rideout et al. 2018). The primers were removed by trimming the first 19bp from all the reads, then we trimmed the forward reads at position 280bp and the reverse reads at position 240bp after inspecting the quality profiles. The forward and reverse reads were then merged (removing the non-matching pairs), screened for chimera and actual sequence variants (ASVs) assigned. Taxonomy was assigned to ASVs using a custom trained classifier (Bokulich, Kaehler et al. 2018) against the SILVA 132 database (Quast, Pruesse et al. 2012), following removal of mitochondrial, eukaryote and chloroplast sequences and resulting in 7,792,427 good-quality sequences. Reads were subsampled at an equal depth (3,364), prior to calculation

of metrics of alpha (Shannon diversity, Chao1 richness) and beta diversity (Bray-Curtis dissimilarity) within QIIME based on ASV-level assignment. Additionally, read counts were obtained for family-level analyses within QIIME.

Statistical analysis

We used R version 4.03 (R Core Team 2020) for all analyses. We employed generalised linear mixed effects modelling (GLMM) with the *lme4* (Bates, Mächler et al. 2015) and *lmerTest* (Kuznetsova, Brockhoff et al. 2017) packages to assess the effects of diet and time since first feeding on various response variables using tank identity as a random factor to account for lack of statistical independence. A gaussian family link was used to model changes in fish size, condition factor and microbiome alpha diversity (Shannon diversity, Chao1 richness), a binomial link to model survival, and a poisson link to assess relative bacterial counts (reads). We employed the *anova* command and the Likelihood Ratio Test (LRT) to determine the significance of random and fixed factors against a null model with no predictors. Starting with a full model containing all main effects, we used the step and drop1 functions to arrive at a minimal adequate model based on changes in AICc (Ligges 2009). We used the *vegan* package (Oksanen, Blanchet et al. 2017) to analyse variation in beta diversity, and employed non-metric multidimensional scaling (NMDS) ordination to visualise Bray-Curtis dissimilarities at days 21 and 94. Multivariate statistical analysis of the gut microbial community was performed by PERMANOVA using *adonis* in the *vegan* package using time since first feeding, diet and fish size as predictors. We used the *Deseq2* package (Love, Anders et al. 2014), to identify significant differences in pairwise ASV comparisons across diet treatments using adjusted probabilities to correct for multiple comparisons ($P_{adj} < 0.05$).

Ethical statement

The study was conducted following approval by Swansea University Animal Welfare and Ethical Review Body (Permit SU-Ethics-Student-300919/1210).

3.4 Results

Variation in fatty acid composition of experimental diets

Analysis of fatty acids (FA) revealed substantial differences in the abundance of omega-3 PUFA in the experimental diets (**Table 3.2**). The amount of EPA was highest in the FISH diet (1.42%) and lowest in the plant diet (0.62%). DHA was particularly high in the microalgae diets and increased with the level of algal oil replacement, being highest in the 100% algae (13.92%) and lowest in the plant diet (0.35%). The DHA:EPA ratio varied almost x30 fold, from 0.56 for the plant diet to 16.77 for the 100% algae replacement. The plant diet, on the other hand, had highest amount of linoleic acid C18:2 (n-6) and polyunsaturated fatty acids.

Table 3.2. Fatty acids profile (%) of the six experimental diets for juvenile Nile tilapia

Fatty acid composition	CON	FISH	PLANT	ALGAE33	ALGAE66	ALGAE100
C08:0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C10:0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C11:0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C12:0	<0.05	<0.05	<0.05	<0.05	<0.05	0.05
C13:0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C14:0	0.82	1.2	0.48	0.81	0.75	0.71
C14:1	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C15:0	0.09	0.12	0.07	0.09	0.1	0.1
C15:1	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C16:0	10.25	10.17	10.58	11	12.34	13.32
C16:1	0.99	1.42	0.59	0.94	0.82	0.74
C17:0	0.12	0.13	0.11	0.12	0.11	0.1
C17:1	0.21	0.23	0.2	0.17	0.2	0.17
C18:0	4.02	3.83	4.15	4.02	3.78	3.6
C18:1	23.67	26.55	20.82	22.42	19.52	17.66
C18:2	29.63	23.47	35.87	27.02	23.5	20.11
C18:3	23.75	22.96	23.41	24.04	23.29	22.37
C18:4	0.2	0.31	0.09	0.18	0.17	0.17
C20:0	0.26	0.25	0.27	0.26	0.26	0.26
C20:1	0.91	1.59	0.29	0.77	0.43	0.23
C20:4	0.12	0.19	0.11	<0.05	0.11	0.12
C20:5 (EPA)	1.03	1.42	0.62	0.95	0.86	0.83
C22:0	0.24	<0.05	0.29	<0.05	0.21	0.24
C22:1	0.36	0.73	<0.05	0.27	<0.05	<0.05
C22:4	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
C22:5	0.24	0.49	0.08	0.23	0.22	0.14
C22:6 (DHA)	1	1.8	0.35	3.95	9.27	13.92
C24:0	<0.05	0.09	0.12	0.09	0.13	0.12
Free FA of extracted fat	3.8	4.2	4.6	3.9	4.9	28.6
Monosaturated FA	26.14	30.52	21.9	24.57	20.97	18.8
Polyunsaturated FA	55.97	50.64	60.53	56.37	57.42	57.66
Saturated FA	15.8	15.81	16.08	16.39	17.71	18.5
Unidentified FA	2.09	3.03	1.49	2.67	3.9	5.04

Effects of diet on survival

Mean survival at the end of the ~3 month feeding trial was 85% (SE = 3.18). Tank identity had a significant effect on survival (chi-square = 52.37, df = 1, $P < 0.001$) with survival ranging from 55% to 99% across tanks. However, survival did not differ across diets once variation between tanks was statistically accounted for (chi-square = 10.61, df = 7, $P = 0.156$).

Effects of diet on growth

Tank identity did not influence variation in fish weight (chi-square = 0.245, df = 1, $P = 0.620$) and therefore a linear model was used to examine growth in weight instead of a mixed effect model. Weight gain depended on time since first feeding ($F_{1,330} = 621.16$, $P < 0.001$), diet ($F_{5,330} = 13.01$, $P < 0.001$) and the interaction between time and diet ($F_{5,330} = 10.91$, $P < 0.001$) as fish fed on the full algal diet (A100) grew much faster than fish fed on the control or plant diets (**Fig. 3.1**). The final weight at the end of the experiment differed significantly between diets ($F_{5,66} = 5.08$, $P < 0.001$). Post-hoc pairwise comparisons (Turkey HSD) indicated that fish fed the full 100% algae diet were significantly larger (mean = 3.36g ± 0.416 SE) than fish fed the plant diet (mean = 1.57g ± 0.249 SE; $P = 0.003$), the control diet (mean = 1.65g ± 0.201 SE; $P = 0.005$) or the 66% algae diet (mean = 1.96g ± 0.277 SE; $P = 0.035$). No significant difference in Fulton's body condition factor (K) was detected between diets at the end of the trial ($F_{5,66} = 1.22$, $P = 0.310$).

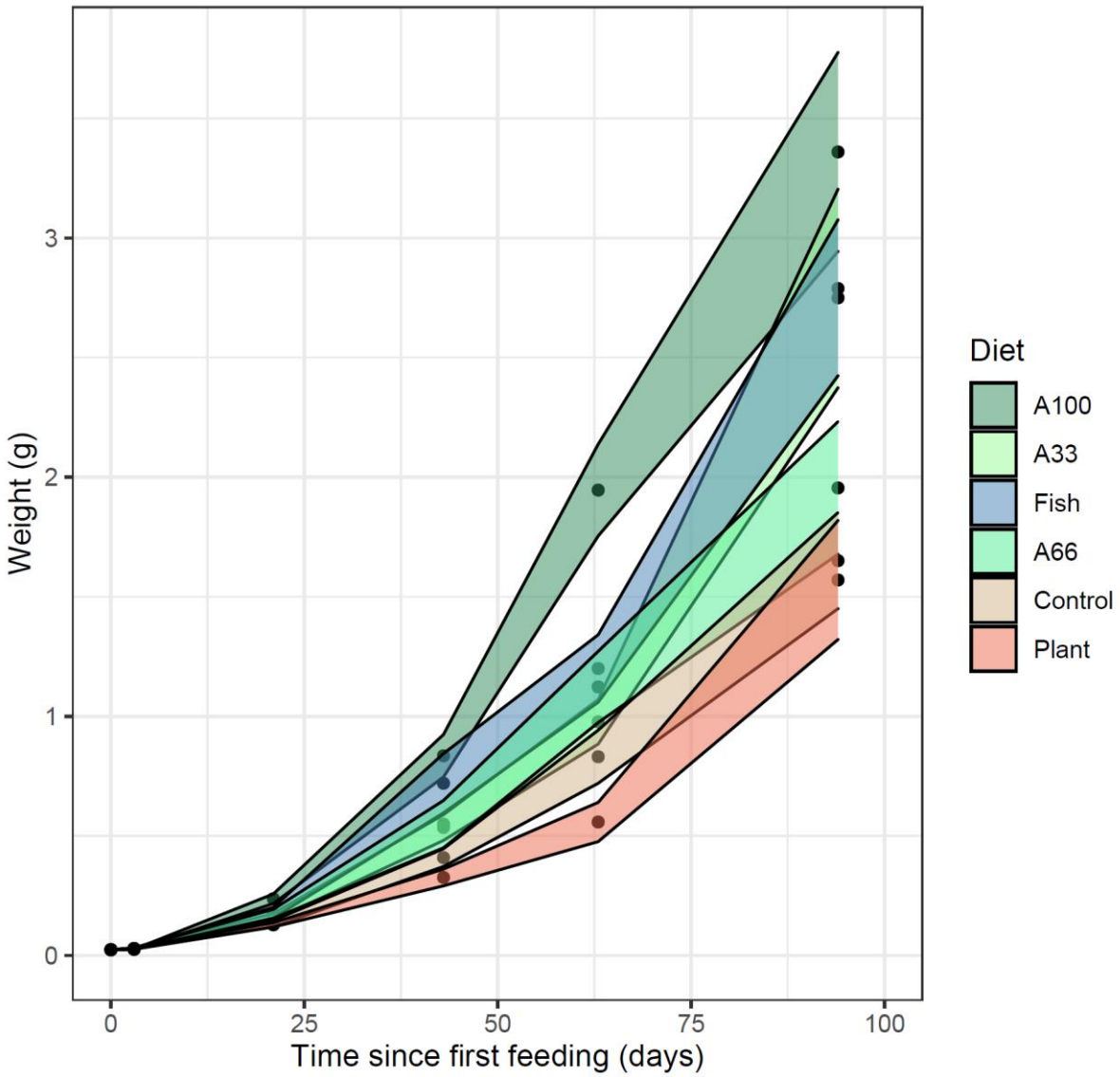


Figure 3.1. Weight gain (g, mean ± SE) of Nile tilapia fed six experimental diets that varied only in the origin of dietary oil (Plant, Fish, Algae) over a 94-day feeding trial. The control (reference) diet had 50% plant oil and 50% fish oil.

Relationship between dietary omega-3 content and deposition in the fish fillet

The omega-3 content of the fat in the fish fillet was closely linked to the omega-3 content in the diet ($F_{1,4}$ = 30.08, P = 0.005; R^2_{adj} = 0.853; **Fig. 3.2**). Fish fed the 100% algae diet, which had the highest omega-3 content (mean = 14.75%), accumulated the highest amount of omega-3 in the fillet (mean = 28.52%), whereas fish fed the plant diet, which was poorest in omega-3 (mean = 0.97%), had the lowest omega-3 content (mean = 15.30%). Inspection of regression coefficients indicates that a 1% increase in omega-3 in the diet increased the omega-3 content of the fat in the fish fillet by 0.86% (SE = 0.158).

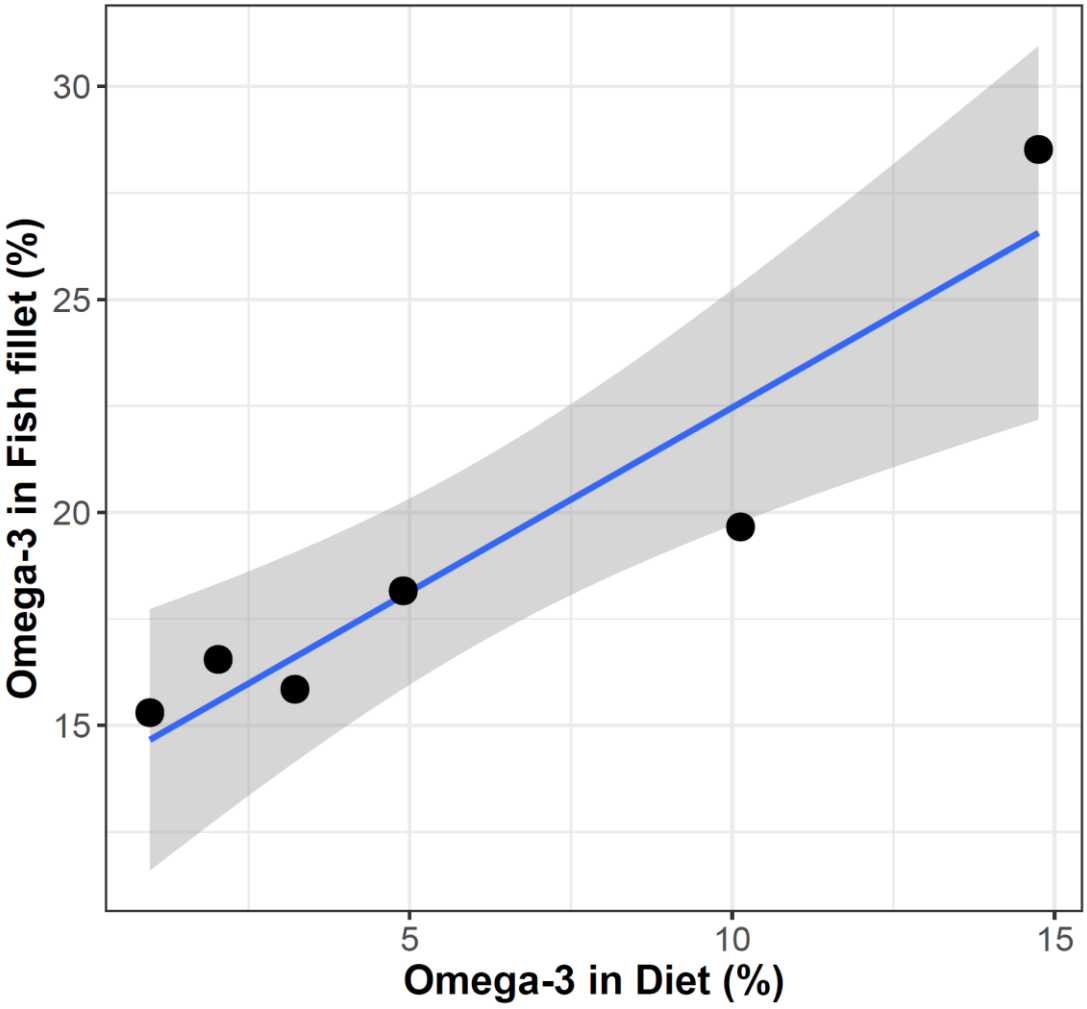


Figure 3.2. Relationship between dietary omega-3 content and omega-3 deposition in the fat of the fish fillet. Each point represents a pooled sample of three fish for each experimental diet and the grey envelope the 95 CI.

Temporal variation in alpha and beta diversity of the gut microbiome

A marked loss of alpha microbial diversity was detected from day 21 to day 94 (**Fig. 3.3**), but this was unrelated to diet (Shannon diversity $P = 0.267$; Chao1 richness $P = 0.588$) or to growth in weight (Shannon diversity $P = 0.668$; Chao1 richness $P = 0.683$). Time elapsing since first feeding was the only significant predictor of alpha microbial diversity in the gut of Nile tilapia, as the microbiome of fish became less diverse over time (**Fig. 3.3a -** Shannon diversity: Time estimate = -0.023, SE = 0.002, $t_{121.58} = -10.92$, $P < 0.001$; **Fig. 3.3b -** Chao1 richness: Time estimate = -0.307, SE = 0.113, $t_{121.79} = -2.79$, $P = 0.007$). Analysis of beta diversity using PERMANOVA on Bray-Curtis dissimilarity distances reached a similar conclusion. Time since first feeding was a good predictor of beta diversity ($F_{1,127} = 224.30$, $P = 0.001$), while diet ($F_{5,127} = 1.358$, $P = 0.201$) and fish size ($F_{1,127} = 0.964$, $P = 0.870$) had no significant effect **Fig3.4**.

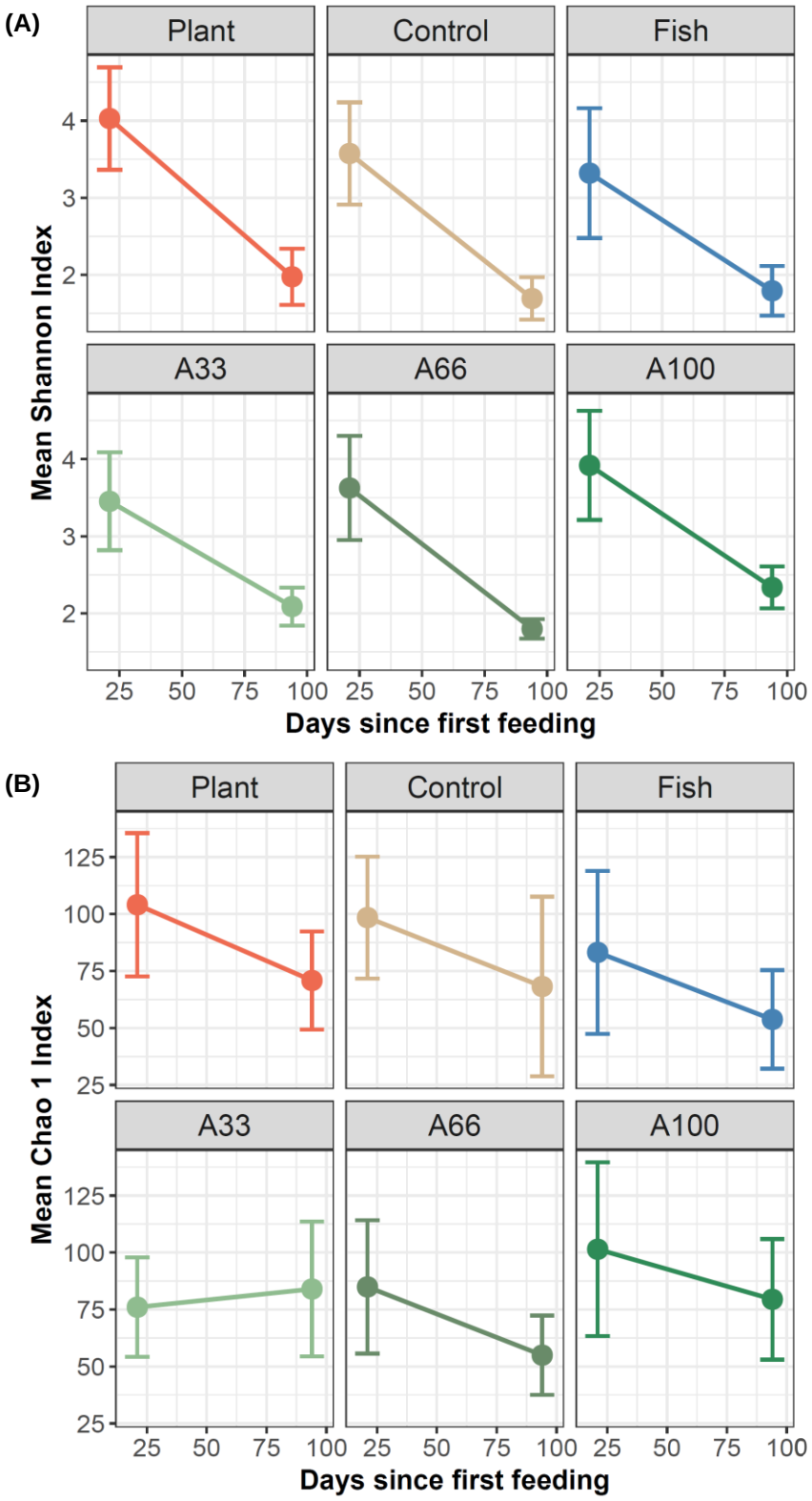


Figure 3.3. Changes in microbiome alpha diversity of Nile tilapia fed on six different diets at day 21 and day 94 of the feeding trial. (A) Shannon diversity (B) Chao 1 richness.

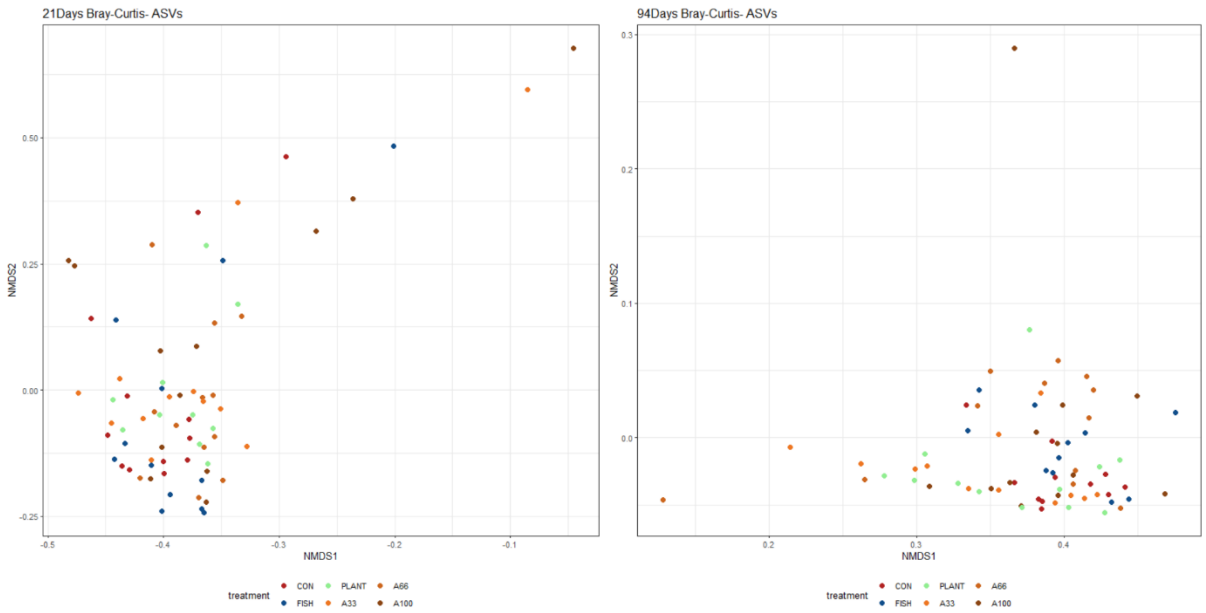


Figure 3.4. Changes in microbiome beta diversity of Nile tilapia fed on six different diets at day 21 and day 94 of the feeding trial. The plots did not reveal significant dissimilarity between the gut bacterial communities of the Nile tilapias (*Oreochromis niloticus*) fed on the six diets at both day 21 and day 94

Variation in the composition of the gut microbiome

The most abundant ASVs in the gut microbiome of Nile tilapia changed substantially over time (**Fig. 3.5)**. At day 21, five ASVs (two from the family Enterobacteriaceae, two different *Aeromonas* spp. and one *Pseudomonas* spp.) dominated the bacterial gut community of Nile tilapia, accounting for ~60% of the total abundance. In contrast, at day 94, the gut microbiome was dominated almost exclusively (90%) by five different ASVs, consisting of two different *Cetobacterium* spp., one *Romboutsia* spp., one from the family Roseiflexaceae, and one from the family Enterobacteriaceae (**Fig. 3.5**).

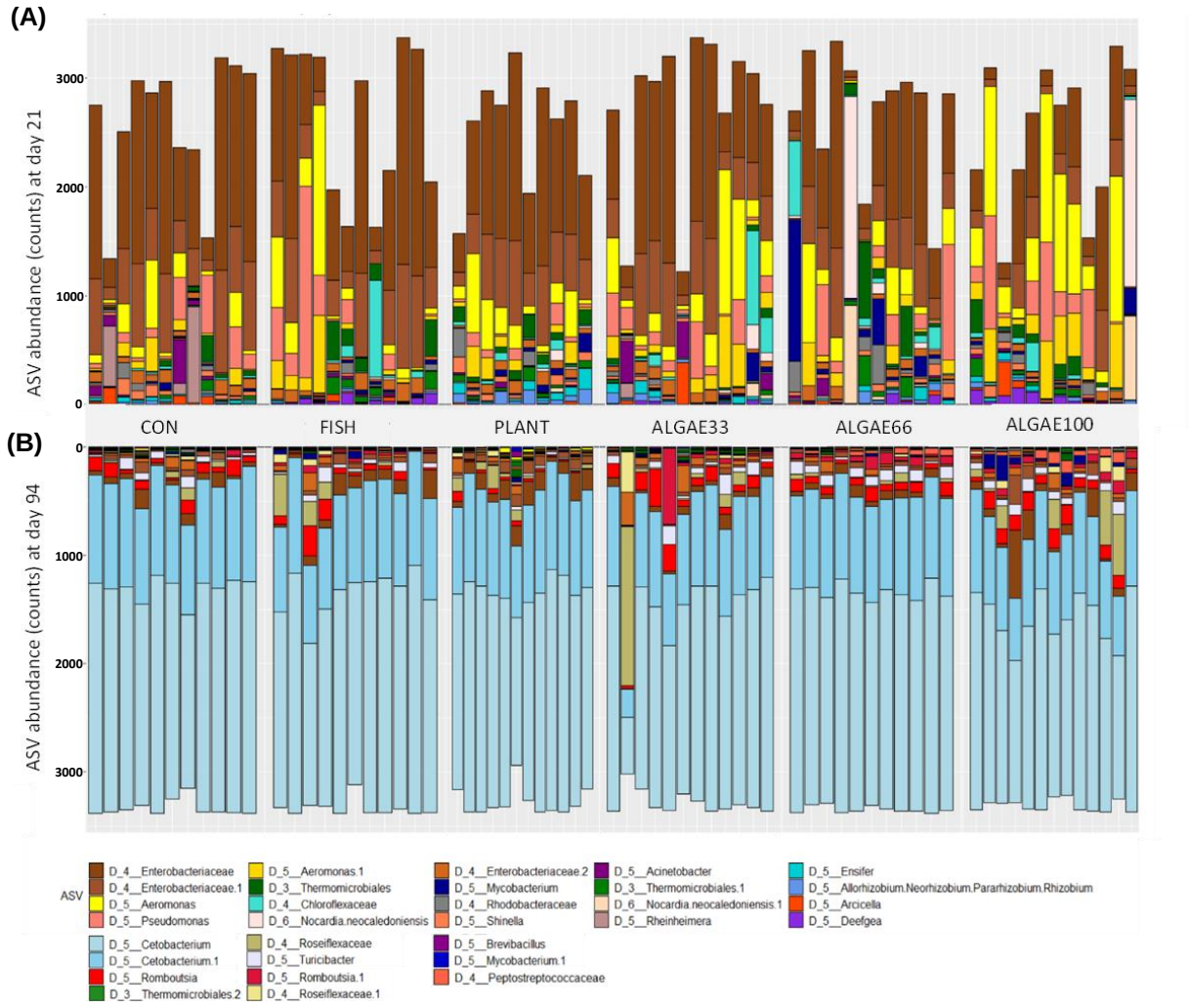


Figure 3.5. Relative abundance (read counts) of the top 20 ASVs at day 21 (top, A) and day 94 (bottom, B) of Nile tilapia fed on six different diets since first feeding. Each bar represents the ASVs of an individual with a level of classification denoted by D_3 (Order), D_4 (Family), D_5 (Genus) and D_6 (Species).

Effect of diet on the gut microbiome composition

At the end of the three month feeding trial, 63 pairwise diet comparisons for ASV abundance were statistically significant after controlling for multiple tests (**Table 3.3**). Most of the differences in bacterial communities were between the fish fed the plant and the three microalgae diets. Across all diets, the ASVs that differed the most in abundance belonged to three bacterial families: Aeromonadaceae, Peptostreptococcaceae and Mycobacteriaceae (**Table 3.4**). Therefore, specific targeted tests were performed at family level.

Table 3.3. Number of ASVs that differed significantly in abundance ($P_{adj} < 0.05$) across pairwise diet comparisons at the end of the feeding trial (day 94).

Diet	***Diet***					
	Plant	Fish	Control	Algae33	Algae66	Algae100
Plant	--	--	--	--	--	--
Fish	13	--	--	--	--	--
Control	2	0	--	--	--	--
Algae33	7	0	1	--	--	--
Algae66	13	1	1	1	--	--
Algae100	10	3	4	4	3	--

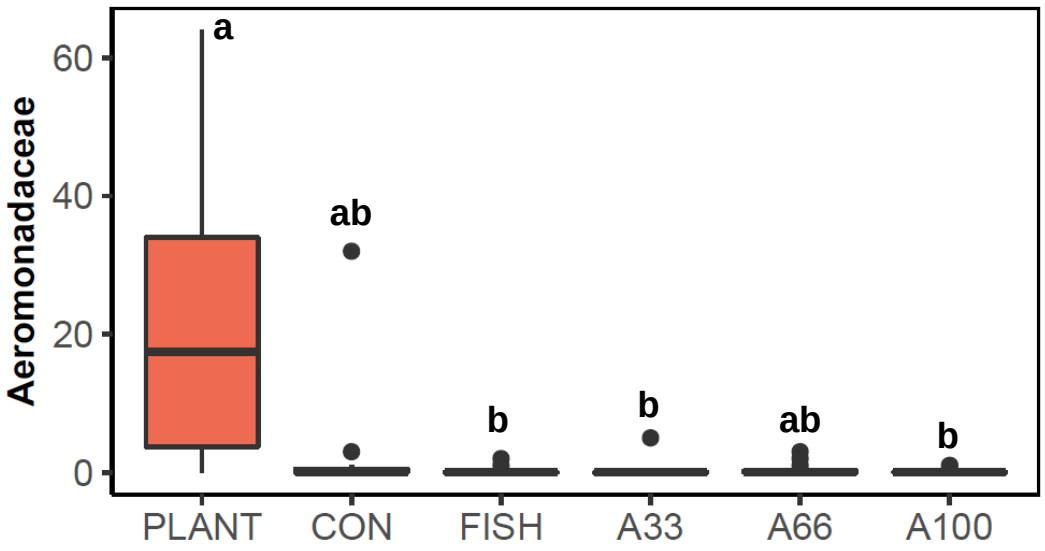
Table 3.4. Bacterial families that differed the most in abundance ($P_{adj} < 0.05$) across pairwise diet comparisons at the end of the feeding trial (day 94).

Diet	***Diet***					
	Plant	Fish	Control	Algae33	Algae66	Algae100
Plant	--	--	--	--	--	--
Fish	Aero	--	--	--	--	--
Control	Aero	0	--	--	--	--
Algae33	Pep	0	Pep	--	--	--
Algae66	Pep	Pep	Pep	Pep	--	--
Algae100	Pep	Pep	Pep	Pep	Myco	--

Aero = Aeromonadaceae; Pep = Peptostreptococcaceae; Myco = Mycobacteriaceae

The overall abundance of Aeromonadaceae differed significantly between diets (LRT, chi-square = 12.65, npar = 5, $P = 0.027$) and increased with fish weight (LRT, chi-square = 21.72, npar = 1, $P < 0.001$). Pairwise Tukey contrasts with Bonferroni adjustment for multiple comparisons indicated that Aeromonadaceae was highest among fish fed plant oil and lowest among fish fed algal and fish oils (**Fig. 3.6a**). The abundance of Peptostreptococcaceae also differed significantly between diets (LRT, chi-square = 22.02, npar = 5, $P < 0.001$) and varied with fish weight (LRT, chi-square = 40.49, npar = 1, $P < 0.001$). Pairwise comparisons revealed that Peptostreptococcaceae was highest among fish fed algal and fish oils, increasing with the level of algal oil inclusion, and lowest among fish fed plant oil ($P < 0.001$; **Fig. 3.6b**). Mycobacteriaceae abundance varied with diet (LRT, chi-square = 11.42, npar = 5, $P = 0.044$) and fish weight (LRT, chi-square = 59.04, npar = 1, $P < 0.001$). They were most abundant among fish enriched with the 100% micro-algae oil and least abundant among fish fed the control diet ($P < 0.05$; **Fig. 3.6c**).

(A)


Aeromonadaceae
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40
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PLANT
CON
FISH
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A66
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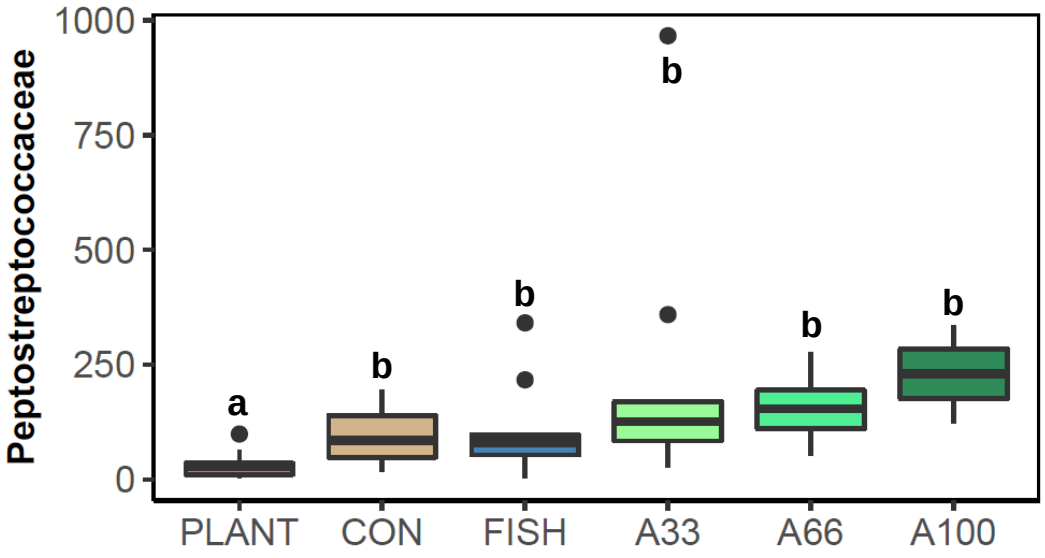
(B)


Peptostreptococcaceae
1000
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PLANT
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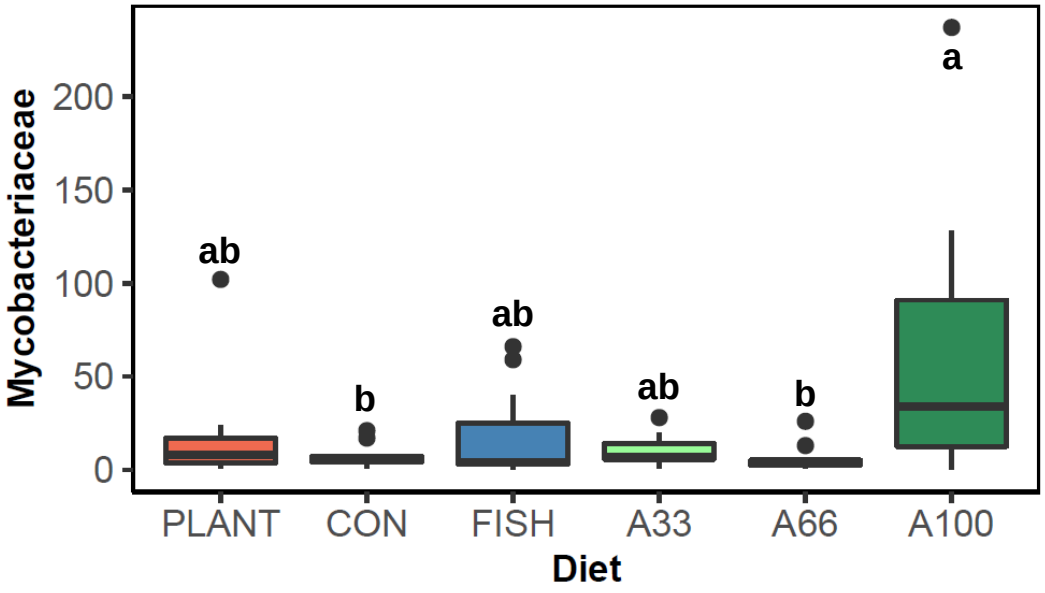
(C)


Mycobacteriaceae
200
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100
50
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a
PLANT
CON
FISH
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A100
Diet

Figure 3.6. Bacterial families that differed in relative abundance (read counts) across pairwise diet comparisons at the end of the feeding trial (day 94). (A) Aeromonadaceae; (B) Peptostreptococcaceae; (C) Mycobacteriaceae. Diets that differ significantly in read counts at $P_{adj} < 0.05$ have different subscripts.

3.5 Discussion

Our study indicates that the type of dietary oil consumed from first feeding through the first three months of development has dramatic effects on the growth, omega-3 fillet content, and composition of the gut microbiome of Nile tilapia. Tilapia raised since first feeding on diet where all the oil originated from the microalgae *Schizochytrium* grew twice as fast than fish fed with plant oil or a mixture of plant and fish oil. A strong positive relation was found between omega-3 content in the diet and the omega-3 content of the fish fillet. Thus, tilapia fed a diet with 100% algal oil had almost twice as much omega-3 in the fat of the fish fillet than fish fed plant oil. A 1% increase in omega-3 in the diet resulted in a ~0.9% increase in omega-3 in the fish fillet, suggesting that there is considerable scope for making Nile tilapia more nutritious using microalgae, as described in a recent study (dos Santos, Schorer et al. 2019).

The early life stage is a critical period for the development of the gut microbiota, with potentially long-lasting consequences for vertebrate health (Tamburini, Shen et al. 2016). Previous work on the effects of the microalgae *Schizochytrium* on the gut microbiome of fish focused on older individuals, whose microbiome may have already been established (Lyons, Turnbull et al. 2017, de Souza, de Lima et al. 2020, Katerina, Berge et al. 2020, Sagaram, Gaikwad et al. 2021), as first feeding induces a substantial change and maturation of the gut microbiota (Ingerslev, von Gersdorff Jørgensen et al. 2014). By conducting our study from first feeding, we were able to reveal the role of diet in the early development of the gut microbiome without the confounding effects of variation in microbial colonization history.

Our results show that the microbiome of Nile tilapia changes rapidly over the first months of life, becoming less diverse as fish develop. Three weeks after the start of the experiment, the gut microbiome of Nile tilapia was dominated by Enterobacteriaceae, *Aeromonas* spp. And *Pseudomonas* spp., as reported previously for several species, including Nile tilapia (Giatsis,

Sipkema et al. 2015), pikeperch (*Sander lucioperca*) (Dulski, Zakęś et al. 2018), yellowtail kingfish (*Seriola landi*) (Wilkes Walburn, Wemheuer et al. 2019) and white cachama (*Piaractus brachypomus*) (Castañeda-Monsalve, Junca et al. 2019).

As the fish develop and begin to feed, the gut microbiome becomes less diverse and stabilises (Ingerslev, von Gersdorff Jørgensen et al. 2014, Giatsis, Sipkema et al. 2015). Consistent with this, we observed a reduction in diversity and richness, and a significant shift in community composition towards a gut microbiome dominated by Fusobacteriaceae, Peptostreptococcaceae and Enterobacteriaceae three months after first feeding. A high abundance of Fusobacteriaceae in the gut microbiome of juvenile fish has been reported previously for Nile tilapia (Adeoye, Yomla et al. 2016) and other warm water fish inhabiting both freshwater and brackish environments (Castañeda-Monsalve, Junca et al. 2019, Nayak, Al Ashhab et al. 2020) and it has been proposed this may form the core microbiome of non-carnivorous fish (Nayak, Al Ashhab et al. 2020). Fusobacteria are able to degrade complex dietary fibres though anaerobic fermentation (Vital, Howe et al. 2014) and may confer benefits to omnivorous and herbivorous hosts (Martin-Gallausiaux, Béguet-Crespel et al. 2018, Castañeda-Monsalve, Junca et al. 2019).

The tendency for the fish gut microbiome to become less diverse, and presumably more stable, over time does not appear to be contingent on a particular diet, as it was observed in our study across six diets that varied widely in oil source and omega-3 content. Diet had no significant effects on alpha microbiome diversity, as reported by others (Adeoye, Yomla et al. 2016), but it altered the composition of the microbiome of Nile tilapia in important ways. In particular, fish fed microalgae diets displayed a high relative abundance of bacteria belonging to the family Peptostreptococcaceae and a low incidence of bacteria belonging to the family Aeromonadaceae.

The family Aeromonadaceae dominates the microbiota of many freshwater fish (Kashinskaya, Simonov et al. 2018, Butt and Volkoff 2019, Shang, Ren et al. 2021), but also includes a large number of opportunistically pathogenic species, such as *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Aeromonas veronii*. An increase in the abundance of *Aeromonas* spp. Has been associated with acute stress (Uren Webster, Consuegra et al. 2020) and compromised health in Atlantic salmon (Wang, Sun et al. 2018). Here, we observed a marked increase in the relative abundance of *Aeromonas* spp. And the family Aeromonadaceae in tilapia fed the 100% plant (soya) oil, which is consistent with a stress response. Nutritional stress resulting from feeding on diets containing only vegetable oil has previously been found to impair growth in Nile tilapia (Betiku, Barrows et al. 2016), and cause intestinal inflammation and increase

susceptibility to disease in other fish species (Booman, Forster et al. 2018, Coronado, Solis et al. 2019, Kiron, Park et al. 2020). These findings suggest that the use of vegetable oils, which are naturally deficient in polyunsaturated fatty acids (arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid), promote the growth of bacteria typically associated with gut disorders in many vertebrates (Villamil, Huerlimann et al. 2018), including humans (David, Maurice et al. 2014) and fish (Austin and Austin 2016, Zhou, Ringø et al. 2018) even if freshwater fish have some capacity for synthesis of omega-3 fatty acids.

In contrast, inclusion of the microalgae *Schizochytrium* in the diet appears to be beneficial, improving growth not only in Nile tilapia (Sarker, Gamble et al. 2016, dos Santos, Schorer et al. 2019, de Souza, de Lima et al. 2020) but also in other species, such as seabream (Ganuza, Benítez-Santana et al. 2008), channel catfish (Li, Robinson et al. 2009) and Atlantic salmon (Kousoulaki, Mørkøre et al. 2016). Unlike previous studies - which used whole microalgae that made it difficult to tease apart the benefits of various microalgal components (Liu, Guo et al. 2016, Teuling, Wierenga et al. 2019), our study shows that inclusion of microalgae oil is sufficient to improve growth.

The abundance of Peptostreptococcaceae also differed significantly between fish fed different diets, being lowest among fish fed plant oil and highest among fish fed fish and algal oil, increasing with the level of algal oil inclusion. The administration of plant-based oils has been reported to lower the abundance of Peptostreptococcaceae in Atlantic salmon (Hartviksen, Vecino et al. 2014, Egerton, Wan et al. 2020), and the same appears to be true for Nile tilapia. Peptostreptococcaceae seems to thrive in anaerobic environments rich in saturated and unsaturated straight-chain C12–C19 fatty acids (Gerritsen, Umanets et al. 2018, Li, Liu et al. 2018). Further studies on the potential relation between Peptostreptococcaceae abundance and omega-3 content seem warranted, as are studies that elucidate the functional role of this family in the fish gut microbiota and how it may influence fish health.

The third bacterial family that differed significantly among fish fed different diets was Mycobacteriaceae, a family common in the microbiota of many fish (Francis-Floyd 2011), including tilapia (Manrique, Figueiredo et al. 2019).

Mycobacteriaceae was most abundant in fish fed the 100% microalgal oil diet, but whether this confers any benefits is uncertain. As for the Peptostreptococcaceae family, some members of the *Mycobacterium* genus are also potentially pathogenic (Hashish, Merwad et al. 2018, Vega-López 2020), which serves to emphasize the dangers of drawing conclusions at family level. The mechanisms that promote the growth of some bacteria in the fish gut and the suppression of others are not clear (Ringø, Olsen et al. 2010), but understanding responses to dietary change

at species (or even strain) level are typically necessary to predict effects on gut health (Gentile and Weir 2018).

3.6 Conclusions

Our study shows that a diet rich in vegetable oil, particularly in linoleic acid (C18:2 (n-6), was associated with an increase in *Aeromonas* spp. commonly associated with intestinal inflammation in the fish gut especially when other factors like stress or antinutritional factors in the diet are present, while a diet rich in DHA (from fish and microalgae oils) promoted the proliferation of Peptostreptococcaceae. In addition, a diet rich in microalgal oil (with a high DHA:EPA ratio) resulted in faster growth and higher omega-3 deposition in the fat of the fish fillet. Taken together our results indicate that microalgae oil could serve as a better replacement of fish oil than plant oil, making tilapia more nutritious and sustainable, which might help to combat malnutrition and alleviate poverty in developing countries.

Statement of relevance

The results of this study will pave the way towards the production of more nutritious Nile tilapia using microgae oil replacement, making the industry more sustainable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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CHAPTER 4 - LONG TERM MIGROALGAE OIL SUPPLEMENTATION INCREASES GROWTH AND OMEGA-3 CONTENT OF NILE TILAPIA (*Oreochromis niloticus*)

4.1 Abstract

The long-term nutritional effects of using microalgae oil to replace fish and plant oil in Nile tilapia (*Oreochromis niloticus*) are not well known. For *Schizochytrium* to progress beyond the experimental phase and become an ingredient of commercial tilapia diets, it is necessary to first assess the effects of this algae over the course of a period lasting as long as a commercial farming cycle. In order to detect those effects, a 11-month long feeding trial, from first feeding to adulthood, was carried out using a control diet (50:50 fish and plant oil) and two diets with 66% or 100% oil from the microalgae *Schizochytrium*. Prolonged use of *Schizochytrium* oil in the diet of Nile tilapia increased growth rate and omega-3 deposition in the muscle fat (up to 32.3 ±1.13% of total fat) without any apparent negative effects. The expression of genes related to the production of Fatty Acid Elongase 5 (*elovl5*) and Catalase (*cat*) in the liver was not altered by long-term ingestion of microalgae oil and was similar to that control fish fed 50% plant and 50% fish oil.

4.2 Introduction

By 2025 the aquaculture feed production is predicted to increase to 101.3 million tonnes per year, and this will translate into an higher demand for aquafeed ingredients (Salin, Arun et al. 2018). While marine oils have initially represented the main lipid source in aquafeeds for many years, in the past decades plant oils have been used as replacement for marine ingredients (Gatlin III, Barrows et al. 2007). The use of plant oils does not come without drawbacks, as plant ingredients often do not match the nutritional requirements of most fish species (Sales and Glencross 2011) and are already being used for human and livestock nutrition, which may require an additional expansion of farmlands to the detriment of natural landscapes (Naylor, Hardy et al. 2009). Microalgae oils represent an alternative to plant oils and also tend to be richer in omega-3 fatty acids (especially DHA and EPA) and have a lower carbon footprint than terrestrial crops (Muller-Feuga 2000), in fact microalgae can fix atmospheric carbon dioxide 10-50 times more than plants (Onyeaka, Miri et al. 2021). Microalgae-based oils represent a sustainable and nutritious alternative to marine oils and are likely to become increasing important for the aquafeed market in the next decades, as industrial-scale production of microalgae like *Schizochytrium* is already bridging the gap between supply and demand (Tocher, Betancor et al. 2019). In addition to being nutritious for the fish, *Schizochytrium* can also be sustainably farmed on low-cost mediums using agricultural by-products (Song, Zang et al. 2015, Nguyen, Su et al. 2018).

Most feeding trials carried out so far lasted less than four months, possibly because the cost of *Schizochytrium* oil is highly expensive (Sarker, Gamble et al. 2016, Sarker, Kapuscinski et al. 2016, Sarker, Kapuscinski et al. 2018, de Souza, de Lima et al. 2020), however commercial tilapia are not ready for harvest before six months (Gupta and Acosta 2004). With production costs of *Schizochytrium* decreasing due to technological improvements (Song, Zang et al. 2015), long-term trials will become more feasible but long term use of alternatives to marine oils can cause alterations in the fish physiology and health condition which may have been unnoticed during the few weeks of in lab-trials (Oliva-Teles 2012, Tocher 2015). In-lab feeding trials in the early 2000s demonstrated the feasibility of such plant oils as near-complete replacement for marine oils in Atlantic salmon, supporting their introduction in the commercial diets (Bransden, Carter et al. 2003, Torstensen, Bell et al. 2005). However, there was some indication that plant oil could halve the omega-3 content in the fillet (Sprague, Dick et al. 2016). In addition, long term feeding of ingredients containing high levels of poly-unsaturated fatty

acids like microalgae oils can put fish at risk of peroxidative attack and cause oxidative stress (Oliva-Teles 2012) which is known to disrupt cell homeostasis (Burton and Jauniaux 2011). Long-term studies are thus needed to assess the true potential of novel ingredients in fish nutrition, as short-term studies may be missing important adverse health effects or benefits that may only be evident at longer time scale. Here, *Schizochytrium* oil was used as 66 and 100% replacement of fish and plant oil in the diet of tilapia and the results compared to those of Nile tilapia fed 50:50 plant and fish oil over a a 11-month long feeding trial. The expression in the liver of genes related to fish health and lipid metabolism was quantified and the omega-3 content of the fish fillets determined. We wanted to assess if prolonged ingestion of *Schizochytrium* oil improved the growth and omega-3 content of Nile tilapia without negative impacts on survival or lipid-related metabolic pathways.

4.3 Materials and methods

Ethics

This study was performed with the approval of the Swansea Animal Welfare and Ethical Review Body (AWERB; approval number SU-Ethics-Student-061119/2002).

Dietary formulation and analysis of the omega-3 content of the fillet

The dietary formulation is described in Chapter 3. Briefly, three experimental tilapia diets were formulated according to the nutritional requirements detailed in Royes and Chapman (2003). Diets varied only in terms of oil composition, the remaining ingredients being otherwise identical to control for confounding effects and examine the specific role of lipid source without interference from other nutrients. The control diet included 50% salmon oil and 50% soya oil to represent commercial diets, which usually contain a mix of fish and plant oil, and the two experimental microalgae diets replaced this with an increasing proportion (66, 100%) of oil from the microalgae *Schizochytrium* sp. (Henry Lamotte Oils GmbH, Germany). We used *Schizochytrium* oil instead of the whole microalgae to make it comparable with the other oils. Proximate analysis on the diets was performed by Sciantec Analytical (part of Cawood Scientific, UK). The fatty acid profile of the diets was obtained using the Fatty Acid Methyl Esters (FAMEs) method and is shown in **Table 4.1**. Total fat and omega-3 content of the Nile tilapia fillet from all the fish per was analysed by Campden BRI. The total fat content of the fish was determined using the Weibull-Stoldt method and the Fatty Acid Methyl Esters (FAMEs) method was used to assess the omega-3 (EPA+DHA) content. All the fish per each treatment were analysed in batch due to their small individual size. The results of the analysis for the fat content was reported as grams of fat in 100 gr of fish fillet, the omega-3 content (EPA+DHA) was reported both as grams of omega-3 fatty acids in 100 grams of fish fillet and as percentage of the total fat.

TABLE 4.1. Fatty acids profile (%) of the three experimental diets for the Nile tilapia.

Fatty acid composition	**CON**	**A50**	**A100**
C08:0	<0.05	<0.05	<0.05
C10:0	<0.05	<0.05	<0.05
C11:0	<0.05	<0.05	<0.05
C12:0	<0.05	<0.05	0.05
C13:0	<0.05	<0.05	<0.05
C14:0	0.82	0.75	0.71
C14:1	<0.05	<0.05	<0.05
C15:0	0.09	0.1	0.1
C15:1	<0.05	<0.05	<0.05
C16:0	10.25	12.34	13.32
C16:1	0.99	0.82	0.74
C17:0	0.12	0.11	0.1
C17:1	0.21	0.2	0.17
C18:0	4.02	3.78	3.6
C18:1	23.67	19.52	17.66
C18:2	29.63	23.5	20.11
C18:3	23.75	23.29	22.37
C18:4	0.2	0.17	0.17
C20:0	0.26	0.26	0.26
C20:1	0.91	0.43	0.23
C20:4	0.12	0.11	0.12
C20:5 (EPA)	1.03	0.86	0.83
C22:0	0.24	0.21	0.24
C22:1	0.36	<0.05	<0.05
C22:4	<0.05	<0.05	<0.05
C22:5	0.24	0.22	0.14
C22:6 (DHA)	1	9.27	13.92
C24:0	<0.05	0.13	0.12
Free FA of extracted fat	3.8	4.9	28.6
Monosaturated FA	26.14	20.97	18.8
Polyunsaturated FA	55.97	57.42	57.66
Saturated FA	15.8	17.71	18.5
Unidentified FA	2.09	3.9	5.04

Experiment design and sampling

The experimental design and source of fish are detailed in Chapter 3. Briefly, we sourced mixed-sex three-day-old Nile tilapia silver strain) from Stirling University, originating from three half-sib families (1 male, 3 females). Fish were housed in 9 plastic 25 L tanks connected to a recirculating aquaculture system (RAS) at the Centre for Sustainable Aquaculture Research (Swansea University, Swansea UK). Rearing conditions and water quality were maintained within the optimal range for Nile tilapia (El-Sayed 2013): temperature: 27–28°C; dissolved oxygen > 4.0mg/L; photoperiod 12D:12L). Each tank contained initially 90 fish. Three tanks were randomly assigned to each of three experimental diets. After 94 days the fish were moved in 1500 L fiberglass tanks in duplicates. Fish were fed to satiation three times per day around 5% bodyweight a day. Six fish per tank (12 per dietary group) were humanely sacrificed using an overdose of 2-phenoxyethanol followed by destruction of the brain according to UK Home Office regulations and sampled at day 349 coinciding with the end of the experiment (11 months). The liver of the fish was sampled using sterile dissection and preserved in RNA-later® (QIAGEN N.V.) first at 4C for 24h and then at -23°C. The whole fillet of the fish was also collected and preserved at at -23°C. Fish development was assessed by measuring final weight and length of the fish, standard growth rate (SGR) and condition factor (CF). The health status of the fish was characterized by recording the mortality rate overtime and by estimating the oxidative stress status of the fish via expression level of the gene responsible to produce the catalase enzyme.

Gene expression analysis

Liver samples from the same fish from which fillet fat content was analysed were used for the gene expression analysis. RT-qPCR was used to quantify the hepatic transcription of known genes involved in lipid metabolism and catalase production, chosen on basis of their response to dietary changes in previous studies involving Nile tilapia **Table 4.2**. The target genes were genes involved in the production of catalase (*cat*) (Xie, Zhou et al. 2016), elongation of very long chain fatty acids protein 5 (*elovl5*) (You, Lu et al. 2019) and lipoprotein lipase (*lpl*) (Tian, Wu et al. 2015). As control genes we used genes responsible for beta actin (*β-actin*) and Elongation factor 1-alpha (*ef-1a*). RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. The purity and concentration of the extracted RNA was assessed using a NanoDrop (Thermo Fisher Scientific) spectrophotometer. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA, with a ratio of ~2.0 in the sample accepted as "pure". Starting from an initial 2µg of total RNA treated with RQ1

DNase (Promega, Southampton, UK), the cDNA was synthesised using M-MLV reverse transcriptase (Promega) and random hexamers (MWG-Biotech) according to the manufacturer's instructions. qPCR primers were optimised via a two-step approach with a temperature gradient and a standard curve for each primer pair. The temperature gradient ranged from ~1 ◦C below to ~7 ◦C above the T_a of the primer pair, using two wells per each temperature containing tilapia cDNA and a master mix containing the primer pair. The temperature chosen for each primer pair was the lowest one to yield consistent Cq values between two replicates, with the temperatures being excluded if it led to Cq values above 30. Once the optimal temperature for each primer was chosen from the gradient, we then evaluated the primer efficiency and non-specific amplification via a standard curve. For each primer, 25 wells containing 1:10 dilution series of tilapia cDNA and master mix in triplicates were run by the qPCR programme alongside three negative controls. We only selected primers with an efficiency value of 90-120% and a linear correlation (r^2) between mean Cq and log cDNA dilution of >0.99. The RT-qPCR reaction was performed using 1:8 diluted cDNA in duplicate, each well containing: 5 µl SYBR Green (Bio-Rad Laboratories, Watford, UK), 0.25 µl Forward primer, 0.25 µl Reverse primer, 0.5 µl water, 4 µl 1:8 cDNA. A negative control was also run in triplicate on each plate. Reactions were run on an CFX96 Real-Time System (Bio-Rad Laboratories) under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 5 s followed by 60 °C for 30s (Except for LPL where the temperature was 58.2°C). β-actin and ef-1a genes were used as reference for qPCR data normalization. The relative gene expression level was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and the value obtained denoted the n-fold difference relative to the control genes. All datasets were given in terms of relative mRNA expression as means ± S.D.

TABLE 4.2. The primers used in this study.

Function classifications	**Gene name**	**Forward and reverse primer (5'–3')**	**Tm (°C)**	**GenBank No.**
Internal reference	β-actin	AATGAGAGGTTCCGTTGC GATGCTGTTGTAGGTGGTT	60	KJ126772.1
	ef-1α	CATCAACATCGTGGTCAT GCTTCCTTCTCAAACTTCT	60	NM_001279647.1
Oxidative stress	CAT	ATTATCCTGGCGAATGTG ATGCTCCGTCTTCTTGTA	60	XM_003447521.5
Lipid uptake	LPL	TGCTAATGTGATTGTGGTGGAC GCTGATTTTGTGGTTGGTAAGG	58.2	XM_003443859.2
FA elongation	ELOVL5	GCCATACCTTTGGTGGAAGA AGGGAGCTGTTCTGTGGATG	60	AY170326

Tm= melting temperature of the primer, FA= fatty acids

Statistical analysis

We used R v3.5.1 (R Core Team 2013) for all analyses. We assessed the effect of diet on fish size, SGR, CF, total fat content of the fillet, omega-3 percentage of the fat, genes expression levels and mortality rate using a binomial link, with tank identity as a random factor, using linear mixed effect models (LMM) implemented in the lme4 package (Bates, Sarkar et al. 2007). The package lmerTest (Kuznetsova, Brockhoff et al. 2017) was used to assess models significance. Starting with a full model containing all main effects (fish size, SGR, CF), we used the step and drop1 functions to arrive at a minimal adequate model based on changes in AICc (Crawley 2013). All models were analysed using single factor one way analysis of variance (ANOVA). If significant effect of the diets was detected, then a pairwise Bonferroni post hoc test was also performed in order to detect differences across the diet-groups.

4.4 Results

Variation in survival and growth performance

The mean survival at the end of the experiment was 85%. Tank identity had a significant effect on survival (chi-square = 33.474, df = 1, P < 0.001) with survival ranging from 55% to 99% across tanks. Survival differed across diets once variation between tanks was statistically accounted for (chi-square = 8.012, df = 2, P = 0.018), with fish fed the A50 diet having the highest survival (mean= 96.00% SE=0.76).

75% of the mortalities concentrated during the first three weeks of the experiment and completely ceased after week 13 (**Figure 4.1)**.

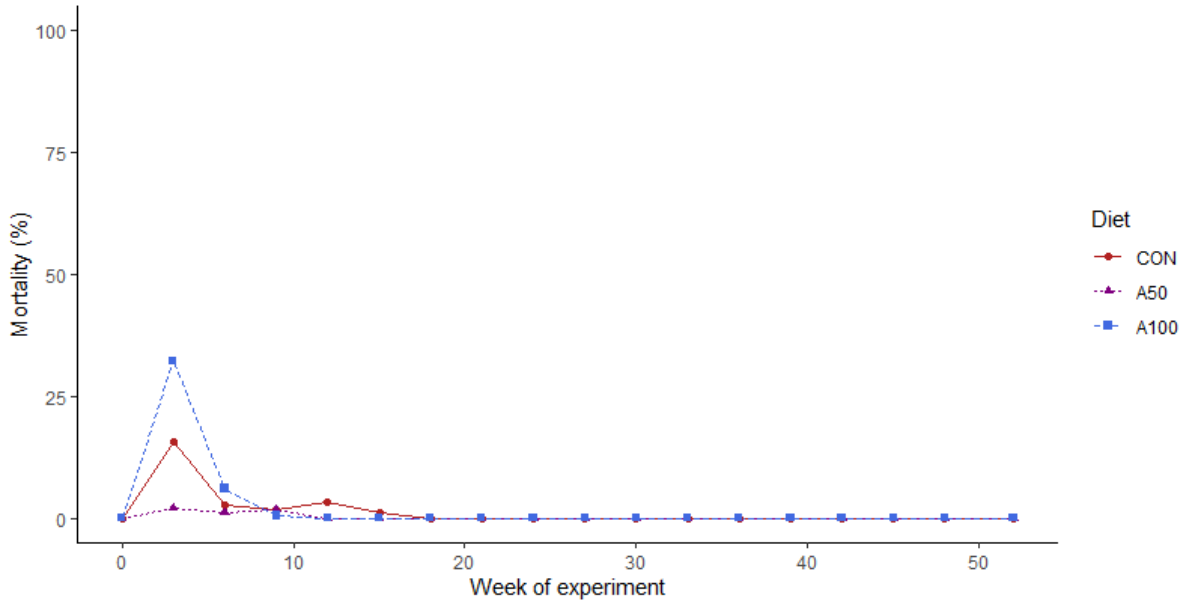

Figure 4.1. Nile tilapia mortality (%) for the duration of the experiment.

Tank identity did not influence variation in fish final weight, final length, SGR and CF (chi-square = 0, df = 1, P = 1); and therefore, a linear model was used to analyse changes in final growth, final length, standard growth rate (SGR) and condition factor (CF).

Weight gain depended on time since first feeding ($F_{1,66}$ = 782.059, P < 0.001), diet ($F_{2,66}$ = 7.0283, P = 0.001) and the interaction between time and diet ($F_{2,66}$ = 6.5702, P = 0.002). The post-hoc pairwise comparisons (Tukey HSD) indicated that the fish fed the A100 diet were significantly larger (mean= 398.56 gr, SE= 28.61) than both the fish fed the A50 diet (mean= 317.01 gr, SE=10.88, P=0.023) and the fish fed the CON diet (mean= 296.24 gr, SE=18.80,P=0.003). Final length was significantly affected by the diet ($F_{2,33}$=4.106, P=0.025), with fish fed the A100 diet being significantly longer diet (mean= 270.5 mm, SE= 6.59) than the fish fed the CON diet (mean= 249.5 mm, SE= 5.47, P= 0.009) but not than fish fed the A50 diet. The diet had a significant effect on the standard growth rate of the fish ($F_{2,33}$=5.449, P=0.009) with fish fed A100 having a significantly higher SGR than the CON fish (P=0.008). The condition factor was not significantly affected by any diet ($F_{2,33}$=0.7171,P=0.378) (**Figure 4. 2).**

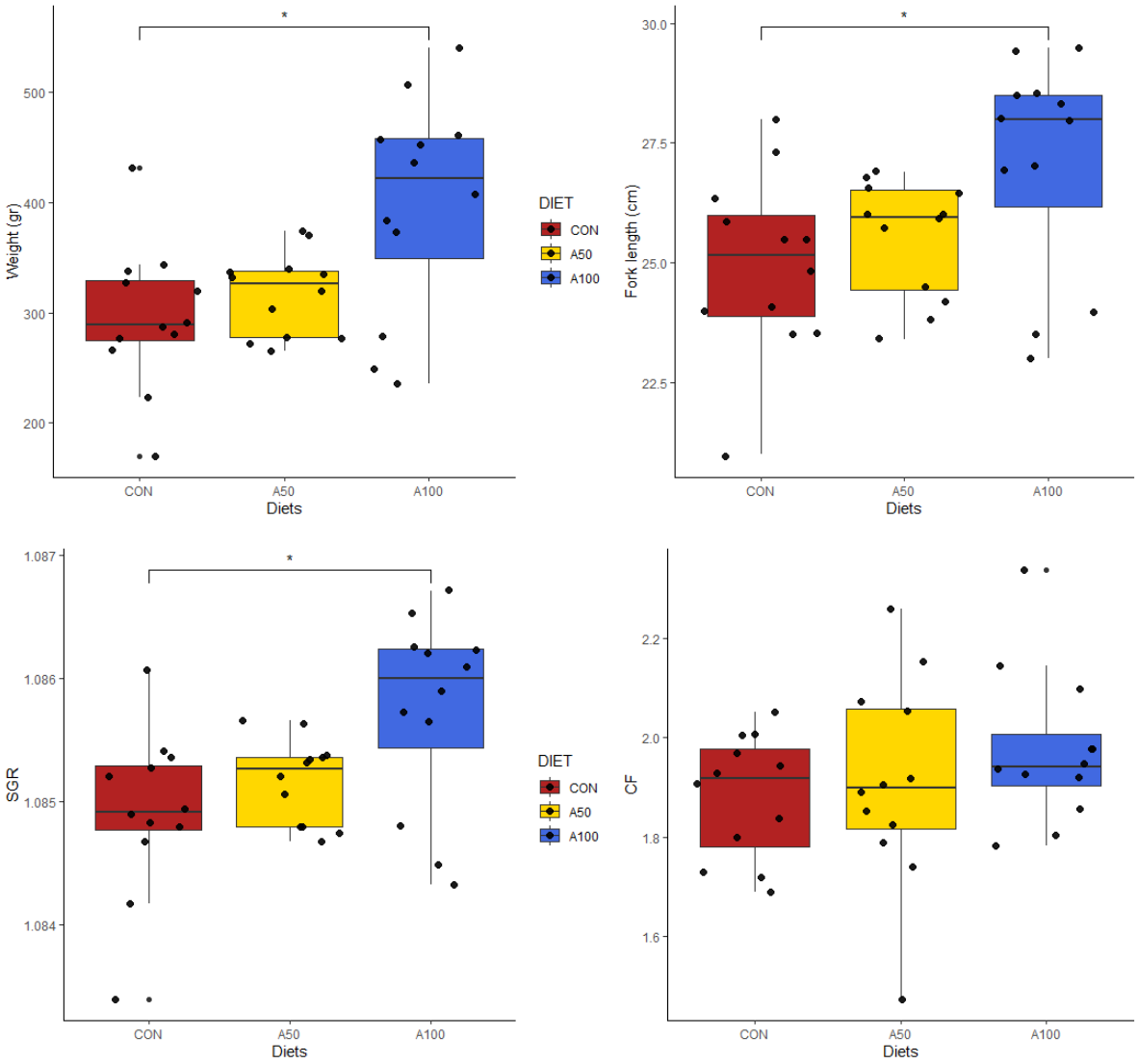

Figure 4.2. Total weight, Fork length, Standard Growth rate (SGR) and Condition Factor (CF) of Nile tilapia (Oreochromis niloticus) at the end of the experiment. Pairwise t test, 2 df , significance levels: * = p<0.05, ** = p<0.01, *** = p<0.001.

Fat content of the fillet and percentage of omega-3 of the fat

The omega-3 content of the fat in the fish fillet was found to be closely correlated to the omega-3 content in the diet ($F_{1,16}$ = 138.21, P <0.005; R_{2adj} = 0.889). and analysis of the regression coefficients indicates that a 1% increase in omega-3 in the diet increased the omega-3 content of the fat in the fish fillet by 0.78% (SE = 0.066). The fish fed the A100 diet had a significantly higher omega-3 content than the fish fed A50 (P<0.001). The fillet of the fish fed CON had an average omega-3 share of the total fat of 21.97± 0.87%, the ones fed A50 26.49±1.31% and the ones fed A100 32.32±1.13% (**Figure 4.3**). Diet had a significant effect on the total fat content of the Nile tilapia fillet ($F_{2,15}$= 3.7589 , P= 0.04751) and on the omega-3 percentage of the total fat ($F_{2,15}$=128.53, P<0.001). The tank in which the fish were housed had no significant effect on the fat content of the fillet and on the omega-3 content of the fat (chi-square = 0, df = 1, P = 1)

The post-hoc pairwise (Tukey HSD) comparison showed that fish fed the CON diet had a significantly lower percentage of omega-3 in the fat than the A50 (P<0.001) and A100 (P<0.001) fish. However, the post-hoc pairwise analysis did not detect a significantly different fat content of the fillet when comparing the CON fish with the A50 fish (P= 0.994) as well as with the A100 fish (P= 0.082).

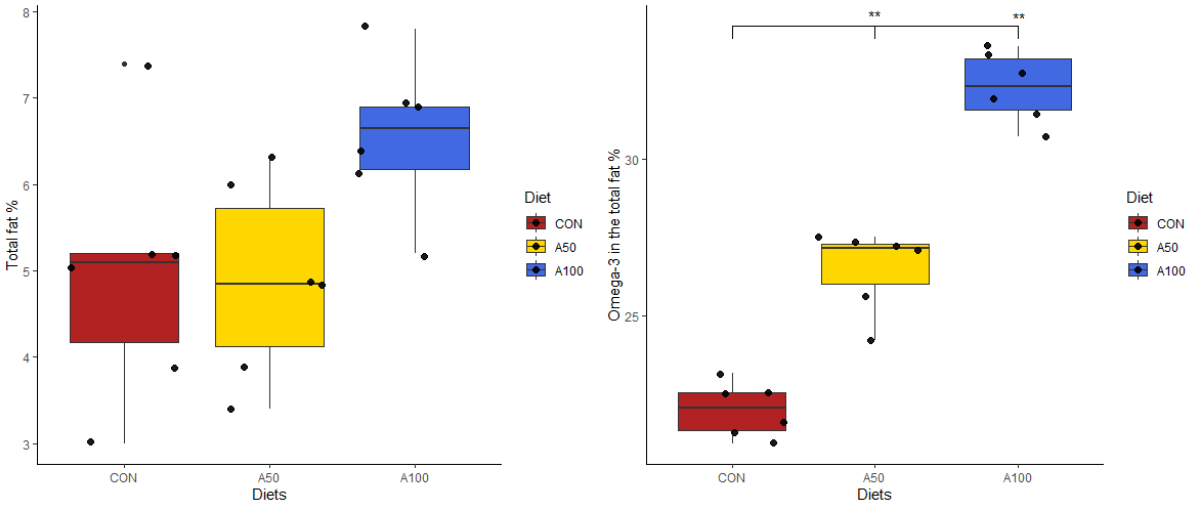

Figure 4.3. Total fat content of the fillet and omega-3 fraction of the total fat in the fillet of Nile tilapia (Oreochromis niloticus) at the end of the experiment. Pairwise t test, 2 df , significance levels: * = p<0.05, ** = p<0.01, *** = p<0.001.

Transcriptional analysis

The mRNA expression levels of *cat* and *elovl5* were not significantly different across treatment groups (**Figure 4.4**) (*cat*: $F_{2,4}$= 3.2424, P= 0.498; *elovl5*: $F_{2,4}$= 0.0145, P= 0.985). The expression levels of lpl was not significantly influenced by the diet either, but this gene was expressed only in 2 out of the total 6 in the CON fish, 2 out of 6 in the A50 fish and 5 out of 6 in the A100 fish, so potentially this gene was switched off. Fish size (*cat*: $F_{1,10}$= 3.9177, P= 0.077; *elovl5*: $F_{1,12}$= 0.071, P= 0.7946), total fat in the fillet (*cat*: $F_{1,11}$= 0.5052, P= 0.492; *elovl5*: $F_{1,11}$= 0.0256, P= 0.876), or omega-3 content of the fillet (*cat*: $F_{1,10}$= 1.1866, P= 0.303; *elovl5*: $F_{1,12}$= 0.0569, P= 0.816) did not affect the expression of any of the target genes.

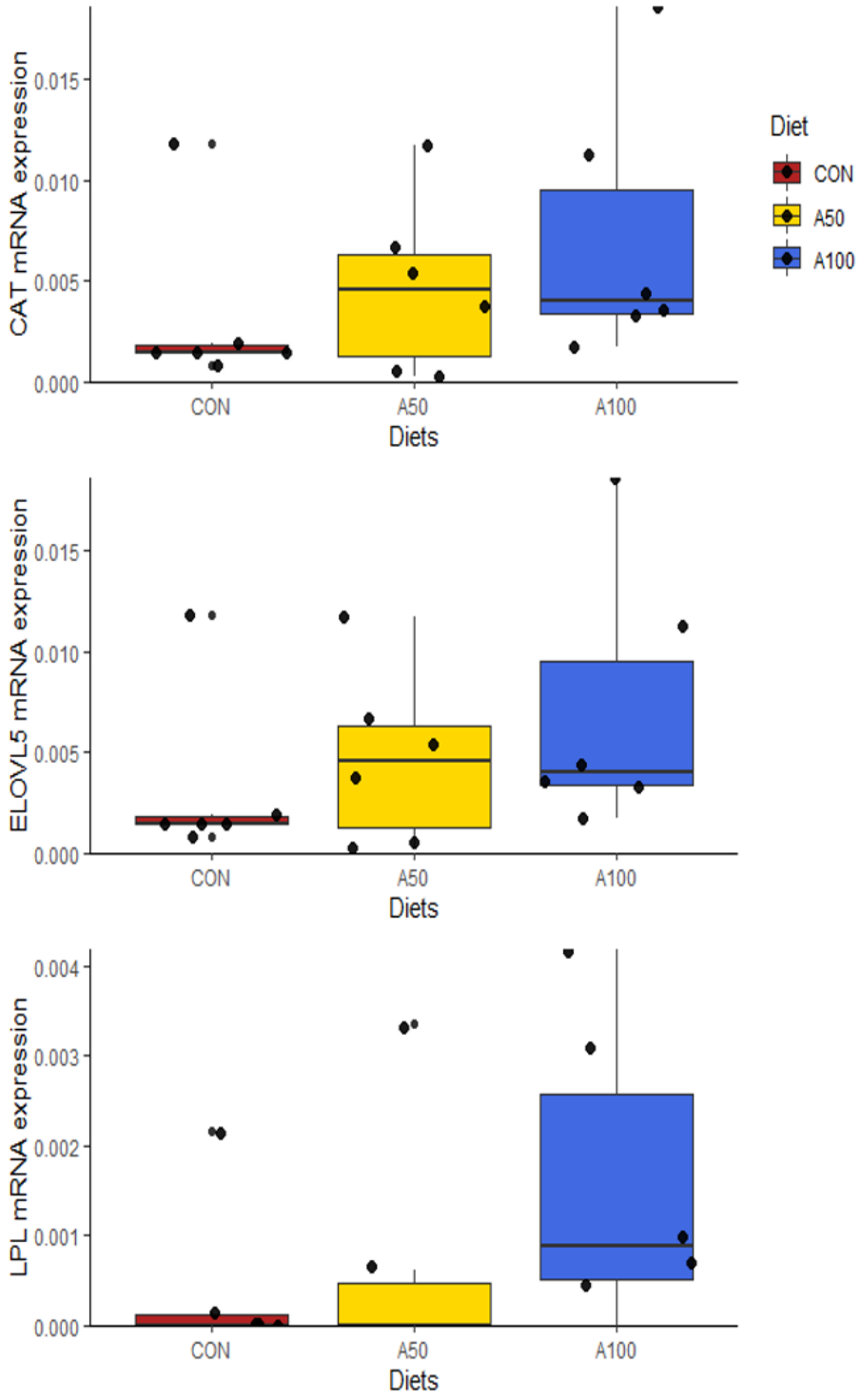

Figure 4.4. mRNA expression of catalase (cat), Elongation of very long chain fatty acids protein 5 (elovl5), Lipoprotein lipase (lpl) in liver of the Nile tilapia (*Oreochromis niloticus*). The mRNA levels of target genes were determined by real time quantitative PCR, using β-actin and ef-1a as a reference genes. Pairwise t test, 2 df , significance levels: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

4.5 Discussion

The use of microalgae as a novel source of lipids in aquaculture feeds has steadily grown over time but considerable uncertainties remain surrounding their efficacy across different fish life-cycle stages (Cottrell, Blanchard et al. 2020). Our study assessed the long-term effect of replacing fish and plant oils with *Schizochytrium* oil in Nile tilapia diets during the entire lifecycle, from first feeding to adulthood.

We found that the positive effects on the growth of Nile tilapia reported by others in short-term studies (dos Santos, Schorer et al. 2019) were maintained after 11 months. Nile tilapia fed a diet with 100% replacement microalgal oil (A100) achieved the largest size at the end of the experiment, grew much faster, and deposit more omega-3 fatty acids than fish fed a control diet with 50:50 plant:fish oils.

The abundance of omega-3 fatty acids has been linked to higher growth rate in farmed fish in previous studies, probably because its function as an essential component of the cellular membrane (Sarker, Gamble et al. 2016). Another remarkable characteristic of the A100 diet was that the proportion of free fatty acids (FFA) was much higher (~29%) than that of the CON diet (~4%) or the A50 diet (~5%) FFA are a natural component of cellular membranes and thanks to their unbound state they can diffuse quickly, making them a readily available energy or building block for cellular development (Stillwell 2016). FFA are less stable than neutral oil (oils in which free fatty acids have been removed via fatty acids neutralisation), which makes them more prone to oxidation (Mahesar, Sherazi et al. 2014) which in turn may led to oxidative stress and inflammation (Piro, Anello et al. 2002, Pohl, Kock et al. 2011). Information on the effects of dietary FFA on the fish is scarce, but red hybrid tilapia (*Oreochromis sp.*) fed a diet rich in FFA from plant origin showed no enhanced growth (Bahurmiz and Ng 2007). Our results suggests that FFA from algal origin enhances growth in Nile tilapia, possibly because it provides a readily available source of additional energy. The current trend in aquaculture nutrition is to produce highly energetic diets to maximise growth, but this can also be detrimental for fish health (Oliva-Teles 2012) as fish can become obese if fed an excessive amount of calories (Chu, Chen et al. 2012, Zang, Shimada et al. 2014). In contrast, the increased growth rate observed in Nile tilapia fed the A100 diet did not resulting in an excessive weight gain (for their length), as the condition factor was not significantly different across diets.

Analysis of the fillet of the Nile tilapia further indicated that the increased growth did not result in excessive fish fattening, as the total fat content of the fillet was not significantly different

across treatments. The quality of the fat was, however, affected by the diets, the amount of omega-3 fatty acids in the fat reflected the omega-3 content of the experimental diets. Previous studies on Nile tilapia have shown thet *Schizochytrium* oil replacement increases the omega-3 content of fish fillet (Sarker, Kapuscinski et al. 2016, dos Santos, Schorer et al. 2019) but the amount of such increase in our study was higher than previously recorded. With an average omega-3 content of 32.3 ±1.13%, the fat Nile tilapia fed A100 had an omega-3 content comparable to Atlantic salmon which can be as high as 35% (Deepika, Vegneshwaran et al. 2014). Nile tilapia fed on A50 had an omega-3 share of the total fat (26.4±1.31%), higher than several marine species like cod, capelin, herrings, sand eels and halibut which have omega-3 levels between 18 and 25% (Pike and Jackson 2010) indicating the possibility of improving the quality of tilapia fillet without the need of completely replacing fish and plant oils with *Schizochytrium* oil. High costs and the difficulty of obtaining a steady supply are the main impediments for using microalgae in aquaculture feeds (Pratiwy and Pratiwi 2020), In this sense, our study shows that the nutritional value of tilapia can be as high as that of marine fish using 66% *Schizochytrium* replacement in aquafeeds.

Schizochytrium oil replacement did not significantly affect the expression of *elovl5* and *lpl* in the liver, which was low across all diets. Tilapia is known to use processes of elongation to convert fatty acids presents in vegetable oils into n-3 Long Chain-PUFA (Tocher, Agaba et al. 2001), a pathway influenced by the expression levels of *elovl5* (Chen, Guan et al. 2018). This bioconversion process is downregulated in diets containing a high concentration of Long Chain-PUFA (Tocher 2015) and thus it is important to better understand if microalgae oil, which is rich in such fatty acids (Ryckebosch, Bruneel et al. 2014), inhibits this natural process of LC-PUFA production in the fish liver. We found that *Schizochytrium* oil, while rich in PUFAs (mainly DHA) did not cause the downregulation of *elovl5*, potentially being one of the factors that led the fish fed the A50 and A100 diets to accumulate more omega-3 PUFAs in the fillet than the fish fed with the normal commercial diet (CON). Another key factor in the incorporation of fatty acids in the tissue is the *lpl* which acts as a "gate keeper" of this process (Tian, Wen et al. 2013). Apraku, Huang et al. (2019), Aanyu, Betancor et al. (2018) reported that the expression levels of *lpl* in Nile tilapia fed plant-based oils. We found that 5 out 6 fish examined from the A100 diet expressed *lpl* in the liver but only 2 out 6 fish did so in the A50 and CON diets. These results suggest that *Schizochytrium* oil promoted the expression of *lpl*, like plant oils, which would explain the higher accumulation of omega-3 fatty acids in the tissues of the fish fed A100.

Effects on fish health

An elevated accumulation of poly unsaturated omega-3 fatty acids in fish, while increasing the nutritional value of the flesh for human consumption, could potentially be deleterious for fish. Diets high in omega-3 content, in particular DHA of which *Schizochytrium* oil is rich, can cause oxidative stress in Atlantic salmon (Kjær, Todorčević et al. 2008), and long term supplementation of fish oil rich in PUFAs has being linked to oxidative stress and even decreased life span in mice (Tsuduki, Honma et al. 2011). Results for Nile tilapia are contradictory, with some studies indicating that diets rich in polyunsaturated fatty acids reduce oxidative stress (Ng and Chong 2004), while others suggest that a high amount of polyunsaturated fatty acids increase oxidative stress (Boonanuntanasarn, Nakharuthai et al. 2019). We assessed the expression of *cat* in the liver, as this is an established marker for oxidative stress that has been used before to assess the nutritional impact of using novel dietary ingredients (Ogunji, Nimptsch et al. 2007, Rahman, Abdellatief et al. 2017, Liang, Huang et al. 2018, Yilmaz 2019), including microalgae (Abdelkhalek, Eissa et al. 2017, Fadl, El-Habashi et al. 2019). We found no significant difference in the expression of *cat* across diets, suggesting that prolonged ingestion of *Schizochytrium* oil did not increase oxidative stress in Nile tilapia. These results, and the lack of any detrimental effects on survival, suggest that prolonged use of *Schizochytrium* oil as a source of fatty acids has no negative health effects in tilapia. Fatty acid nutritional deficiencies, either due to the lack of essential fatty acids or rancid PUFAs, are known to cause persistent levels of low mortality in fish (Shefat and Karim 2018), but in our study 75% of all mortalities where concentrated during the first three weeks of the experiment, and the survival rate of the fish was in line with values recorded in commercial Nile tilapia hatcheries elsewhere (Boyd, McNevin et al. 2005), where a low mortality is often viewed as a good welfare indicator for farmed fish (Ellis, Berrill et al. 2012)

4.6 Conclusions

Prolonged use of microalgae *Schizochytrium* oil as replacement of plant and marine oil improved the final weight and growth rate of Nile tilapia. While the role played by DHA from microalgae in promoting growth of tilapia is well understood, the effect of free fatty acids as a potential growth-catalyst remains unclear. We found that fish fed *Schizochytrium* oil had significantly higher omega-3 content in their flesh than those fed a commercial diet. The omega-3 content increased with *Schizochytrium* oil inclusion and reached a high level that exceeds what was ben reported in the literature for Nile tilapia previously, without a significant increase in total fat content. The expression of the elongase *elovl5* gene as not significantly affected by the incorporation of *Schizochytrium* oil, unlike that reported for fish fed solely on plant oils. Finally, no indication of oxidative stress (expression levels of *cat*) and nutritional deficiencies were detected across diets, suggesting that *Schizochytrium* oil inclusion had no long-term harmful effects on Nile tilapia. Therefore, our results indicate that *Schizochytrium* oil can be used as a long term replacement of plant and fish oil, improving the growth rate of Nile tilapia without any apparent negative health effects.

CHAPTER 5 – EFFECTS OF MICROALGAE SCHIZOCHYTRIUM OIL ON THE GROWTH PERFORMANCE AND OMEGA-3 CONTENT OF ENDANGERED MANYARA TILAPIA (*Oreochromis amphimelas*)

5.1 Abstract

Captive breeding can be used to support efforts to protect fish biodiversity, mainly by breeding fish for restocking and, potentially, also by making available local species for fish farming, making non-native species redundant. A good candidate for captive breeding for conservation is the endangered Manyara tilapia (*Oreochromis amphimelas*) currently living only in Tanzania. Like many novel species in aquaculture, knowledge on the dietary requirements is limited, and this represents one of the main challenges in a breeding programme for conservation purposes. To address these issues, we assessed the growth, fat and omega-3 content of the fillet, and gonad maturation of Manyara tilapia fed on microalgae oil based diets which were previously successfully used in its close relative, the Nile tilapia (*Oreochromis niloticus*).

Like in Nile tilapia, 66% partial replacement of fish and plant oil with microalgae oil did not increased fish size but it increased deposition of omega-3 in the fillet by 7.99%. Complete replacement (100%) with *Schizochytrium* oil enhanced growth of Manyara tilapia by 29.3% and its omega-3 content by 13.71%. No difference in fat content or ovary maturation was detected among diets

Microalgae-fed Manyara tilapia had an omega-3 fat content than many marine fish, and represent a local and sustainable source of omega-3 fatty acids to combat malnutrition in Tanzania

5.2 Introduction

Fish nutritional requirements change alongside the life stage of the fish, with sexual maturation inducing reallocations of nutrients within the body and changes of feeding habits (Cejas, Almansa et al. 2004). During the ovaries' maturation stage, female fish require higher amounts of omega-3 and omega-6 fatty acids in their diet as they are a key component of for the development of the egg's yolk (Rennie, Huntingford et al. 2005). Omega-3 and omega-6 fatty acids become the building blocks of the neural system and cell membranes of the developing embryos (Glencross 2009). Traditionally, omega-3 and omega-6 fatty acids were obtained from marine sources but in recent years alternative (mostly vegetable) oils have proven effective in partially replace marine oils without compromising fish performances as long as the nutritional requirements of the species were respected (Agh, Jafari et al. 2019)

However, the exact nutritional requirements and formulated diets are available only for a limited number of species (Webster and Lim 2002) and this may compromise the reliable production of healthy fish which is a major constraint in aquaculture operations, especially for novel species in aquaculture (Izquierdo, Fernandez-Palacios et al. 2001). Aquaculture is also becoming an effective tool for conservation of endangered species by breeding them in captivity and then releasing them in the wild for restocking (Froehlich, Gentry et al. 2017). Aquaculture techniques previously used in the caviar industry were used to breed and successfully re-introduce the previously locally extinct Baltic sturgeon (*Acipenser oxyrinchus*) in Germany which had disappeared from the Baltic Sea since 1996 (Gessner, Arndt et al. 2011). In Italy, captive breeding programmes have seen initial success in producing juveniles of critically endangered *Squalius lucumonis* for restocking the waters of the Latium region (Tancioni, Martinoli et al. 2019) after its population was decimated by the introduction of non-native species (Cerri, Ciappelli et al. 2018). Perhaps the most promising example of coupling fish farming and nature conservation is represented by the efforts put in place to guarantee a future to the Mexican Totoaba (*Totoaba macdonaldi*) after fishing and poaching reduced the numbers of this species to a critically endangered status (Juarez, Konietzko et al. 2016). While many conservation projects have focused only on the restocking of endangered species, this conservation project went a step further by promoting the development of a sustainable commercial aquaculture of this species (Aznar 2010, Mata-Sotres, Lazo et al. 2015). A key for

the success of the establishment of a captive totoaba population was the in-depth research in the nutritional requirements of this species as at the beginning no specific diet was available for this fish (Minjarez-Osorio, González-Félix et al. 2012, Villanueva-Gutiérrez, González-Félix et al. 2020). The lessons learned by the conservation effort of totoaba could be applied to protect an endangered and potentially new species in aquaculture, the Manyara tilapia (*Oreochromis amphimelas*).

Manyara tilapia is an IUCN endangered species native to shallow lakes in central Tanzania. It is heavily fished and threatened by the introduction of exotic tilapines, including *Oreochromis esculentus, Oreochromis niloticus, Oreochromis leucostictus and Coptodon spp*. (Shechonge, Ngatunga et al. 2019), which can displace the smaller Manyara tilapia from its usual habitat via antagonistic behaviour (Champneys, Genner et al. 2020). As the species is only present in three lakes in Tanzania (Shechonge, Ngatunga et al. 2019), establishing a spawning captive population could help future conservation efforts through captive breeding. In addition, the culture of Manyara tilapia could be important not only for reintroduction into the wild, but also for sustainable commercial aquaculture, as it requires less space and matures faster than its larger cousin, which may ease pressure on the fisheries of lake Manyara which are already compromised by unsustainable human activities causing mass mortalities in wild fish stocks (Nonga, Mdegela et al. 2010). Yet, as for many species new to aquaculture, one of the main constraints in establishing a reproductive captive population of Manyara tilapia is the lack of knowledge regarding its nutrition.

Manyara tilapia is closely related to the larger Nile tilapia (*Oreochromis niloticus*) which is well studied due to its high economic relevance and worldwide distribution for farming, fishing, and bio-control purposes (Canonico, Arthington et al. 2005). An abundance of highly poly unsaturated fatty acids (PUFAs) in the diet of Nile tilapia diet is key to promote growth, enhance its nutritional value facilitate sexual development (El-Sayed, Mansour et al. 2005), and the same is probably true for Manyara tilapia.

While marine oils have been the main sources of PUFAs for Nile tilapia diets for many years, microalgae appear to be the most promising alternative source for those ingredients as their production is more sustainable (Koyande, Chew et al. 2019), they can be farmed in areas unsuitable for conventional crops (Charles, Msagati et al. 2019) and are showing to greatly

improve tilapia growth performances and nutritional value when compared with traditional ingredients (dos Santos, Schorer et al. 2019, Sarker, Kapuscinski et al. 2020) without negatively affect hemato-immunological parameters and gut health (de Souza, de Lima et al. 2020).

In **Chapter 3** and **Chapter 4** of this thesis I assessed the replacement of conventional fish and plant oils with oil from microalgae *Schizochytrium* in Nile tilapia diets from first feeding to adulthood. An improvement in growth and omega-3 deposition was observed without any apparent negative effects on the gut microbiota or the hepatic metabolism of the fish. Schizochytrium oil is rich in DHA which is considered important for fish development, especially during neurogenesis (Emery, Norambuena et al. 2016). As the need for DHA appears to be conserved across many farmed fish (Emery, Norambuena et al. 2016), Manyara tilapia could potentially benefit from the incorporation of DHA-rich *Schizochytrium* oil in the diet, as Nile tilapia does.

In this study Manyara tilapia were fed three diets previously used on Nile tilapia that varied only in the level of *Schizochytrium* oil inclusion in it (0, 66 and 100% of the total oil in the diet). The growth , omega-3 content of the fillet, and female gonad maturation were recorded after 3 months.

5.3 Materials and methods

Ethics

This study was performed with the approval of the Swansea Animal Welfare and Ethical Review Body (AWERB; approval number SU-Ethics-Student- 300120/2401).

Dietary formulation and analysis of the omega-3 content of the gutted fish

Three experimental tilapia diets were formulated according to the nutritional requirements detailed in Royes and Chapman (2003) for Nile tilapia as a formulation specific for Manyara tilapia is currently unavailable. Diets varied only in terms of oil composition, the remaining ingredients being identical, to control for confounding effects and examine the specific role of lipid source without interference from other nutrients (**Table 5.1)**.

Table 5.1. Formulation (g/1000g) of the three experimental diets for the Manyara tilapia (*Oreochromis amphimelas*)

	CON	A50	A100
Maize	134	134	134
Wheat	200	200	200
Wheat Bran	80	80	80
Wheat gluten	125	125	125
Line Seed Meal	125	125	125
Fish meal	141	141	141
Soya bean meal	135	135	135
Fish oil	25	12.5	--
Soya oil	25	12.5	--
Schizochytrium oil	--	25	50
Vitamin and mineral premix	10	10	10

The control diet included 50% salmon oil and 50% soya oil to represent commercial diets, which usually contain a mix of fish and plant oil (Ng and Chong 2004), and the two experimental microalgae diets replaced this with an increasing proportion (66, 100%) of oil from the microalgae *Schizochytrium* sp. (Henry Lamotte Oils GmbH, Germany). Proximate analysis on the diets was performed by Sciantec Analytical (part of Cawood Scientific, UK). The fatty acid profile of the diets was obtained via the using the FAMES method and it is shown in **Table 5.2**. We used *Schizochytrium* oil instead of the whole microalgae to directly test the effect of the oil only not other components of the microalgae such as its cell-wall, etc. Analysis on the Manyara tilapia fillets from each tank was performed by Campden BRI, the total fat content was determined via the Weibull-Stoldt method and the percentage of omega-3 present in the total fat was obtained using the FAMES method.

Table 5.2. Fatty acids profile (%) of the three experimental diets for the Manyara tilapia (Oreochromis amphimelas).

Fatty acid composition	CON	A50	A100
C08:0	<0.05	<0.05	<0.05
C10:0	<0.05	<0.05	<0.05
C11:0	<0.05	<0.05	<0.05
C12:0	<0.05	<0.05	0.05
C13:0	<0.05	<0.05	<0.05
C14:0	0.82	0.75	0.71
C14:1	<0.05	<0.05	<0.05
C15:0	0.09	0.1	0.1
C15:1	<0.05	<0.05	<0.05
C16:0	10.25	12.34	13.32
C16:1	0.99	0.82	0.74
C17:0	0.12	0.11	0.1
C17:1	0.21	0.2	0.17
C18:0	4.02	3.78	3.6
C18:1	23.67	19.52	17.66
C18:2	29.63	23.5	20.11
C18:3	23.75	23.29	22.37
C18:4	0.2	0.17	0.17
C20:0	0.26	0.26	0.26
C20:1	0.91	0.43	0.23
C20:4	0.12	0.11	0.12
C20:5 (EPA)	1.03	0.86	0.83
C22:0	0.24	0.21	0.24
C22:1	0.36	<0.05	<0.05
C22:4	<0.05	<0.05	<0.05
C22:5	0.24	0.22	0.14
C22:6 (DHA)	1	9.27	13.92
C24:0	<0.05	0.13	0.12
Free FA of extracted fat	3.8	4.9	28.6
Monosaturated FA	26.14	20.97	18.8
Polyunsaturated FA	55.97	57.42	57.66
Saturated FA	15.8	17.71	18.5
Unidentified FA	2.09	3.9	5.04

Experiment design and sampling

We sourced mixed-sex three-day-old Manyara tilapia (*Oreochromis amphimelas*) from Swansea University, originating from three half-sib families (3 males, 9 females). Fish were housed in 9 plastic 25 L tanks at the Centre for Sustainable Aquaculture Research (Swansea University, Swansea UK). Rearing conditions and water quality were maintained within the optimal range for the closely related Nile tilapia (El-Sayed 2013): Temperature 27–28°C; Dissolved oxygen > 4.0mg/L; Photoperiod 12D:12L; Conductivity >3000 < 4000 µS/ cm; Ammonia (NH_4) < 0.02mg/L; Nitrite (NO_2) < 0.2mg/L; Nitrate (NO_3) < 50mg/L; Water flow 1L/min; Alkalinity 20 and 150 mg/L of $CaCO_3$. Each tank contained initially 50 fish. Three tanks were randomly assigned to each of three experimental diets and fish were fed at satiation their respective diet thrice per day (at 9am, 12pm and 4pm).

Ten fish per tank (30 per dietary group) were humanely sacrificed using an overdose of phenoxyethanol followed by destruction of the brain according to UK Home Office regulations and sampled at the end of week 12 coinciding with the fish reaching sexual maturity (3 months).

The gonads of the fish were sampled and stored in 10% Neutral buffered formalin (NBF). The rest of the body was preserved frozen at at-23 C.

Total length, total weight, liver weight and gonad weight of the fish were recorded both for growth performance analysis, which included Standard Growth Rate (SGR) and Condition factor (CF), and for the calculation of the Gonado-Somatic Index (Hossain, Jewel et al. 2012), Hepato-Somatic Index (Vosylienė and Mikalajūnė 2006).

Histological analysis

Histological preparation was performed by the School of Veterinary Medicine of the University of Glasgow (UK). Gonads from the female fish preserved in 10% NBF were dehydrated in 70–100% ethanol after which they were cleared using xylene before being embedded in wax. All those steps were performed in vacuum and under pressure. The samples were then cut into 2um thick longitudinal sections, in duplicates. Analysis was carried out with an “Olympus BX43" light microscope (Olympus Soft Imaging Solutions) using a cellSens Entry 1.6 (Build 9464)

software. The percentage of cells at various stages of oogenesis (pre-vitellogenic, early vitellogenic, vitellogenic, mature and atretic) was assessed by scoring the cell type in each of the 100 x 40 mm^2 squares of a grid overlying the microscope images (**Figure 1**). The process was performed in duplicates for each sample. The criteria used to score the maturation stages of the oocytes were based on the work Srijunngam and WATTANASIRMKIT (2001) for Nile tilapia.

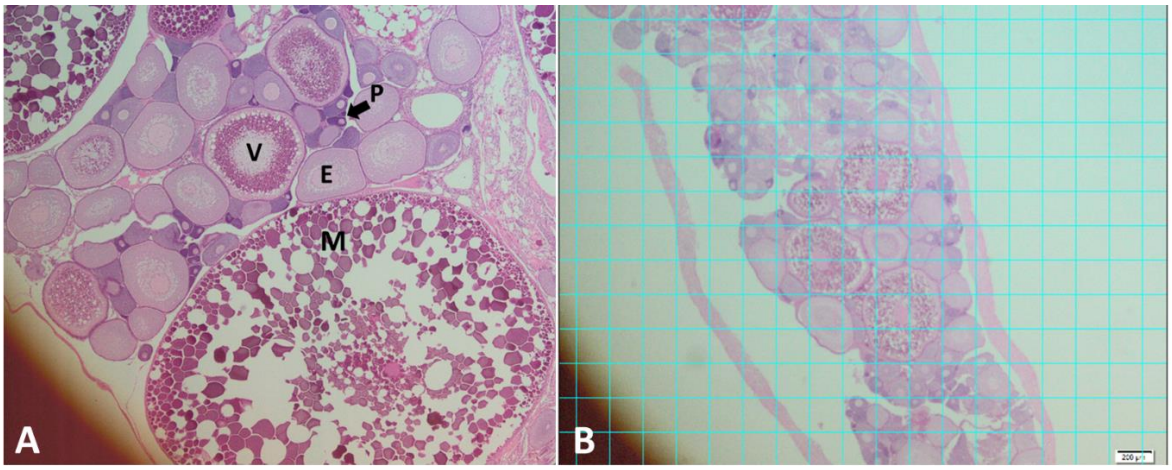

Figure 5.1. A Histological slide of ovaries from Manyara tilapia showing oocytes at pre-vitellogenic (P), early-vitellogenic (E), vitellogenic (V) and mature (M) stages. **B** View of the grid overlapped on the histological slides to assess the percentage of the oocytes at various stages of maturation present in the ovaries

Statistical analysis

We used R v3.5.1 for all analyses. We assessed the effect of diet on fish size, GSI, HSI, total fat content of the gutted fish, omega-3 percentage of the fat and oocyte maturation stage, with tank identity as a random factor, using linear mixed effect models (LMM) with the lmer package. A binomial link was used to model survival. The package lmerTest (Kuznetsova, Brockhoff et al. 2017) was used to assess models significance. Starting with a full model containing all main effects, we used the step and drop1 functions to arrive at a minimal adequate model based on changes in AICC (Crawley 2013) All models were analysed using single factor one way analysis of variance (ANOVA). If significant effect of the diets was detected, then a Post-hoc pairwise comparisons (Turkey HSD) test was also performed in order to detect differences across the diet-groups.

5.4 Results

Growth performances, total fat content and omega-3 content of the fat

The final weight of the fish was significantly affected by the diet ($F_{2,86}$=5.5602, P=0.005) and, to a lesser extent, by the sex of the fish ($F_{1,86}$=6.4027, P= 0.013) but not by the tank in which they were housed (chi-square =0.0229, df = 1, P =0.879) (**Figure 5.2 A**). Post-hoc pairwise comparisons (Turkey HSD) indicated that Fish fed the A100 diet were significantly heavier (mean = 2.78g ± 0.21 SE) than the fish fed the CON (mean = 2.15g ± 0.07 SE, P=0.01) and the A50 (mean = 2.25g ± 0.10 SE, P=0.04) diets. No significant weight difference was detected between the fish fed CON and A50 (P=1.00). The final length of the fish was significantly affected by the diet ($F_{2,86}$=4.2459, P=0.017) and by the sex ($F_{1,86}$=6.0233, P=0.016) but not by the tank (chi-square =0, df = 1, P =1), with the fish fed the A100 diet being longer (mean = 56.43mm ± 1.48 SE) than the CON (mean = 52.63mm ± 0.61 SE, P=0.037) fish and longer than the A50 (mean = 52.76mm ± 0.94 SE, P=0.047) ones (**Figure 2 B**).

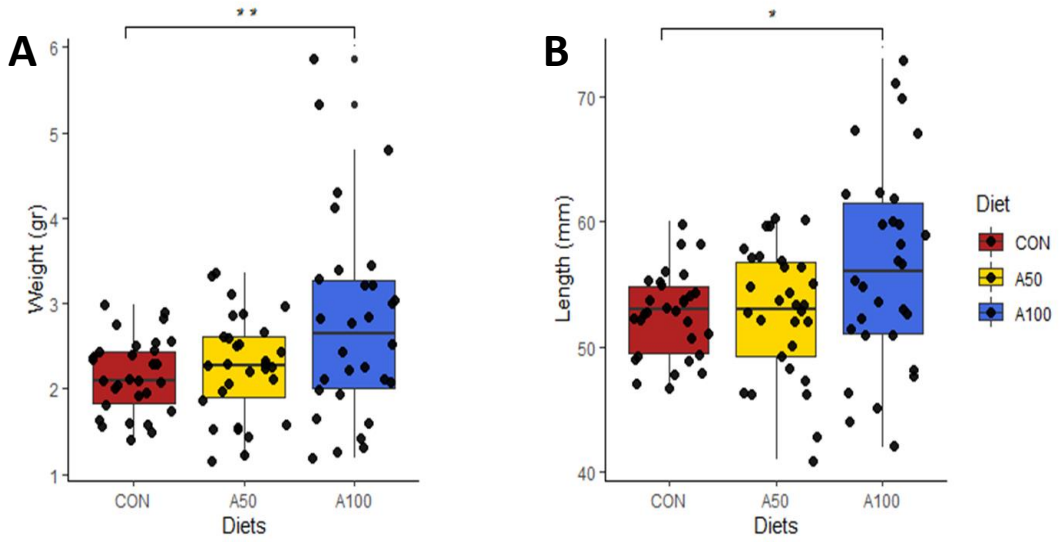

Figure 5.2 Total weight (**A**) and Total length (**B**) of the Manyara tilapia (*Oreochromis amphimelas*) at the end of the experiment. Pairwise t test, 2 df , significance levels: * = p<0.05, ** = p<0.01, *** = p<0.001.

The Standard Growth Rate (SGR) was only significantly affected by the diet ($F_{2,86}$=3.3190, P=0.041) and by the sex of the fish ($F_{1,86}$=5.4086, P=0.022) but not by the tank of the fish (chi-square =0, df = 1, P =1). The post-hoc analysis (Turkey HSD) however did not detect any significant difference across the three diet groups (**Figure 5.3A**). Male fish had a higher SGR than the female ones across all the diets indicating a faster growth rate. The Condition Factor (CF) of the fish was not affected by diet ($F_{2,86}$=1.4681, P= 0.236), sex ($F_{1,86}$=0.1006, P= 0.752) and by the tank used to house the fish (chi-square =0, df = 1, P =1). (**Figure 5.3B**) Mean survival at the end of the ~3 month feeding trial was 97% (SE = 0.61). Tank identity had a significant effect on survival (chi-square = 5.2706, df = 1, P = 0.021) with survival ranging from 93% to 99% across tanks. However, survival did not differ across diets once variation between tanks was statistically accounted for (chi-square = 0.458, df = 2, P = 0.7952).

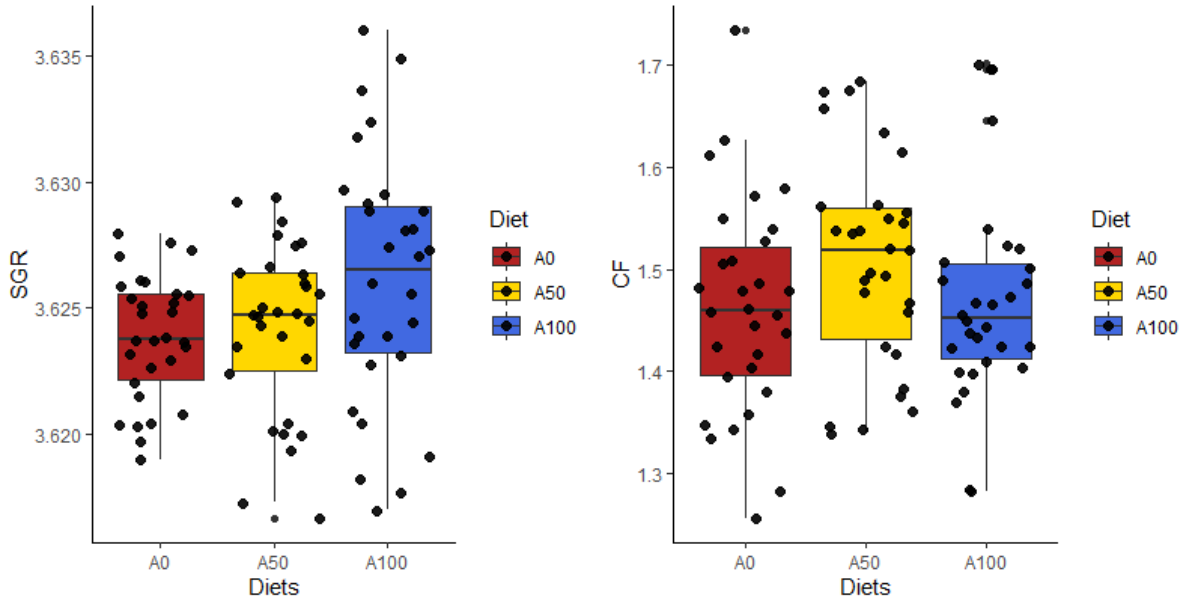

Figure 5.3. Standard Growth rate SGR (**A**) and Condition Factor CF (**B**) of the Manyara tilapia (*Oreochromis amphimelas*) at the end of the experiment.

Diet had no significant effect on the total fat content of the fillet from Manyara tilapia ($F_{2,6}$=0.974, p=0.430) but it had a significant effect on the omega-3 percentage of the total fat ($F_{2,6}$=592.31, p<0.001[7]). The omega-3 content of the fat in the fish fillet was closely linked to the omega-3 content in the diet ($F_{1,7}$ = 824.67, P <0.005; R_{2adj} = 0.990). The post-hoc pairwise comparison showed that the fish fed the CON diet had a significantly lower percentage of omega-3 in the fat than the A50 (P<0.001) and A100 (P<0.001) fish. When comparing A100 and A50, the fish fed the A100 diet had a significantly higher omega-3 content of the fat the fish fed A50 (P<0.001) (**Figure 5.4B**). The fillet of the fish fed CON had an average omega-3 share of the total fat of 18.97± 0.56%, the ones fed A50 26.92±0.57% and the ones fed A100 32.54±0.25% (**Figure 5.4A**). Inspection of regression coefficients indicates that a 1% increase in omega-3 in the diet increased the omega-3 content of the fat in the fish fillet by 1.05% (SE = 0.036)

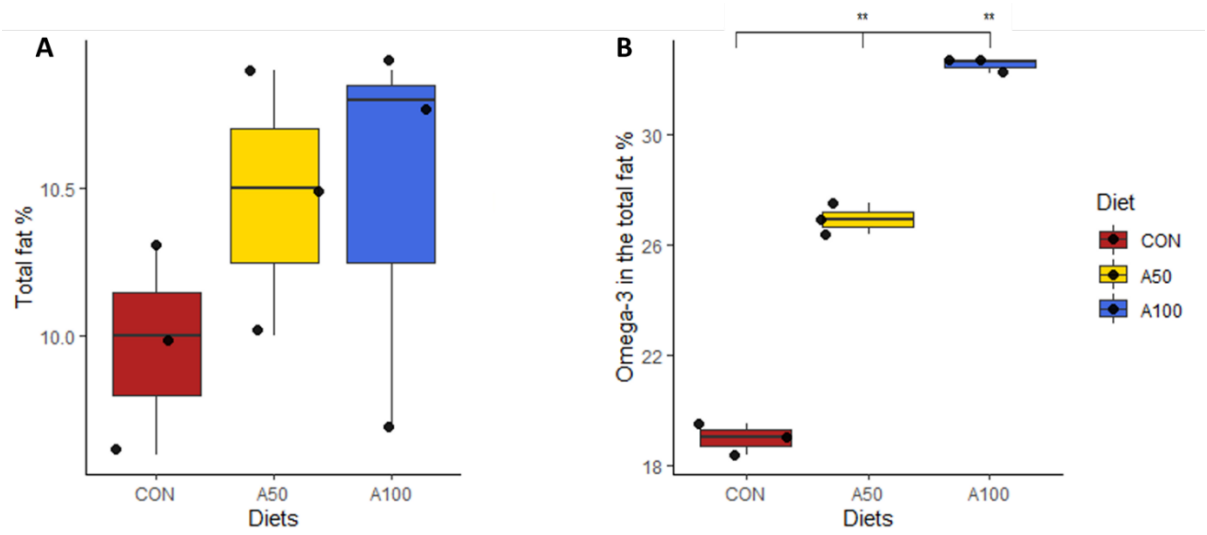

Figure 5.4. Total fat content (**A**) of the fillet and omega-3 fraction of the total fat (**B**) of the fillet of Manyara tilapia (*Oreochromis amphimelas*) at the end of the experiment. Pairwise t test, 2 df , significance levels: * = p<0.05, ** = p<0.01, *** = p<0.001.

Gonadosomatic Index (GSI), Hepatosomatic Index (HSI) and histology of the ovaries

The GSI of the fish was significantly affected by the sex of the fish ($F_{1,85}$=16.043, P<0.001) but not significantly affected by the total weight of the fish ($F_{1,85}$=1.082, P=0.301), by the tank in which the fish were housed (chi-square =2.358, df = 1, P =0.124) and by the diet on which the fish were fed ($F_{2,85}$= 0.1452, P= 0.865). The HSI was significantly affected by the sex of the fish ($F_{1,85}$= 10.724, P= 0.001) and not by the diet ($F_{2,85}$= 2.7253, P= 0.071), by the weight ($F_{1,85}$=0.3700, p=0.544) and by the tank in which the fish were housed (chi-square =0, df = 1, P =1). When looking at the difference between sexes, female fish had a larger GSI and HSI than the males.

Histological analysis of the ovaries revealed that the weight of the fish significantly affected the percentage abundance of the oocytes at early vitellogenic ($F_{1,35}$=9.8374 , P=0.003), vitellogenic ($F_{1,35}$=4.9327 , P=0.033) and mature ($F_{1,35}$=9.9732 , P=0.003) stage. The total length of the fish, diet and the tank in which the fish were housed did not significantly affect the abundance of oocytes at any maturation stage. (**Figure 5.5**)

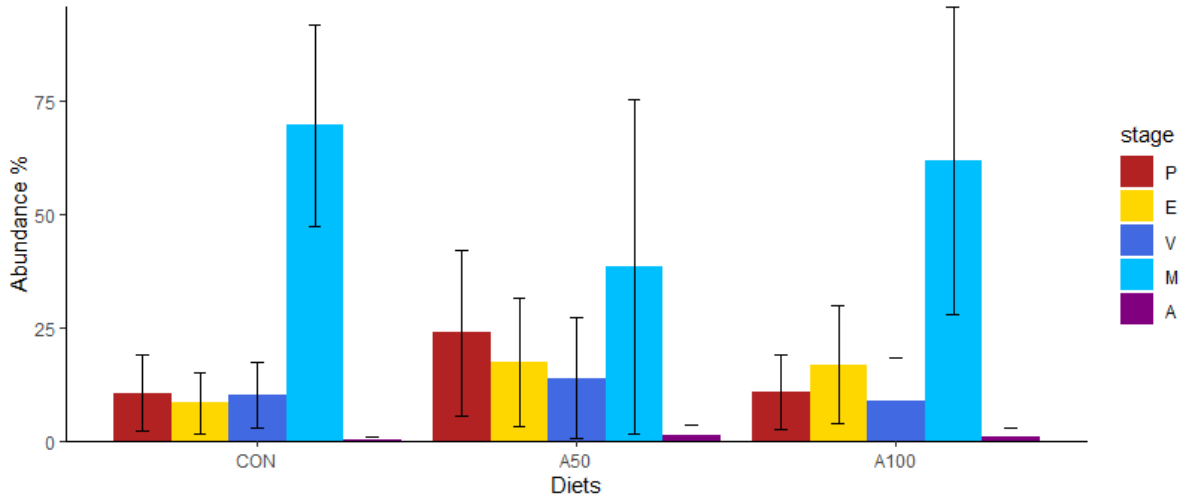

Figure 5.5. Percentage abundance and Standard Deviation of the oocytes of Manyara tilapia (*Oreochromis amphimelas*) at pre-vitellogenic (P), early vitellogenic (E), vitellogenic (V), mature (M) and atretic (A) stage at the end of the experiment.

5.5 Discussion

Poor growth and inability of the fish to reach sexual maturity are the main constraints when it comes to novel species in aquaculture and these issues are often related to inadequate nutrition (Izquierdo, Fernandez-Palacios et al. 2001). Building on previous on Nile tilapia, we assessed the two diets containing *Schizochytrium* oil on the growth, omega-3 content of the fillet and gonad maturation of Manyara tilapia, a species endangered in its native habitat.

Growth performances and Omega-3 content in the flesh

Schizochytrium has previously been reported to improve the growth of Nile tilapia (Sarker, Kapuscinski et al. 2016, dos Santos, Schorer et al. 2019, Sarker, Kapuscinski et al. 2020), But no previous study had assessed the growth of Manyara tilapia, nor its omega-3 content. We tested if this novel ingredient would also have a positive effect on the growth and fatty acid profile of Manyara tilapia. Larger fish with plenty of omega-3 fatty acids in their fat tend to have better reproductive performances due to the surplus of energy stored in larger bodies (McBride, Somarakis et al. 2015) and the abundance of a key player in the vitellogenic processes like omega-3 PUFAs (Johnson 2009). These highly productive Manyara tilapia could potentially form the bulk of a captive breeding population from which obtain high-quality fry for the restocking of lake Manyara.

Manyara tilapia fed the A100 diet were significantly larger than both the CON and A50 fish but had a similar condition factor, which was not significantly different across diets. No difference in size was recorded between fish fed the control diet and fish fed the A50 diet indicating that if enhanced growth is the main target, then complete oil replacement would be needed.

Schizochytrium oil also affected the omega-3 content of Manyara tilapia, and fed the control diet had an omega-3 share of the total fat of 18.97%, which is higher than that of Nile tilapia (16.55% (**Chapter 3** of this thesis), and African catfish (3.2%) fed on commercial diets (Shadyeva, Romanova et al. 2019), the two species most commonly consumed by local people around Lake Manyara (Nonga, Mdegela et al. 2010). Even with just a partial replacement of

fish and plant oil with *Schizochytrium* oil in the A50 diet greatly increased the omega-3 content of the Manyara tilapia (up to 26.92%). This is higher than found for many marine fish species , such as halibut, herring and sand eel which have an omega-3 share of the fat ranging from 18 to 25% (Pike and Jackson 2010). When the totality of fish and plant oil was replaced with *Schizochytrium* oil in A100, the omega-3 content of Manyara tilapia reached 32.68±0.25%, which is close to the 35% shown by Atlantic salmon (Deepika, Vegneshwaran et al. 2014), considered to be the "gold standard" for omega-3 fatty acids content (Sugata, Wiriadi et al. 2019). This increase in omega-3 content due to the use of microalgae oil in the diet was not accompanied by a significant increase in total fat content, which indicates that the fish were more nutritious and yet still lean. In addition to the possible restocking of lake Manyara, the possibility of farming a locally sourced tilapia richer in omega-3 than most marine farmed fish opens interesting possibilities for the local communities in Tanzania and Africa in general in the future. Lack of omega-3 fatty acids in the diet of many African children, especially during weaning and early childhood, has been associated with gastro-intestinal inflammation, disrupted neural development and mental health diseases (Van der Merwe 2010, Charles, Msagati et al. 2019). Across the globe, marine fish are the primary source of omega-3 for human consumption (Ellulu, Khaza'ai et al. 2015) but in mainland Africa logistics make it difficult to prove a reliable supply of marine products (Bennett 2002), making freshwater aquaculture the main source of omega-3 on the continent (Adeleke, Robertson-Andersson et al. 2020). While it is possible to enhance the omega-3 content of Nile tilapia by using *Schizochytrium* oil (dos Santos, Schorer et al. 2019), the use of a local species would be advantageous as it would reduce disease outbreaks associated with semi-intensively mixed-sex tilapia monoculture which is the dominating fish farming practice in Tanzania (Mmanda, Mulokozi et al. 2020), as well as the presence of a invasive non-native species such as Nile tilapia, which can escape from fish farms and outcompete local fish species (Canonico, Arthington et al. 2005).

Comparison between Manyara and Nile tilapia from previous chapters

One of the aims of this study was to compare the results previously obtained with Nile tilapia (**Chapter 2**), under the same culture conditions, with the Manyara tilapia ones to assess the transferability of the benefits from the use of *Schizochytrium* oil across the various species of

the *Oreochromis* genus as many of them have an high economic relevance (Nagl, Tichy et al. 2001).

Comparison of the growth performances revealed that both Nile and Manyara tilapia significantly increased its total weight after three months only when fish and plant oil was completely replaced in the A100 diet. Between fish fed the A50 diet and the control diet there was no difference in final weight in both species. This was the case also when comparing the standard growth rate of the two tilapias species, indicating that in the two species growth was significantly promoted only by full replacement with microalgae oils and partial replacements are not advisable when aiming for an improved growth in those two fish. Similarities in the growth performances may also indicate similar nutritional requirements allowing for Manyara tilapia to be successfully grown to adulthood using Nile tilapia diets. This can represent an advantage for a future establishment of Manyara tilapia in captivity as Nile tilapia feed is available in Tanzania due to the local Nile tilapia farming industry (Kajungiro, Palaiokostas et al. 2019).

When comparing the omega-3 content of the Manyara tilapia with the one of the Nile tilapias fed on the same diets and at the same age from our previous experiment, some trends become evident. The omega-3 content of the fillet increased with the increase of *Schizochytrium* oil in the diets of both species, however Manyara tilapia had a higher omega-3 content of the fat across all treatments including control, indicating a lipid metabolism more efficient in building up omega-3 reserves in the body.

Manyara tilapia fed A50 had a similar omega-3 share of the total fat to the one of the Nile tilapias fed on A100. With an omega-3 share of the total fat of 32.68%, Manyara tilapia fed the A100 diet had 4% more omega-3 in its fillet than the A100 Nile tilapia at the same age. To reach a similar omega-3 content, Nile tilapia had to be fed on A100 for eleven months thus increasing its feeding costs. Enriching Manyara tilapia appear to be more cost efficient, as it required only half of the microalgae oil necessary to achieve similar omega-3 values when compared with Nile tilapia. Using less microalgae oil as possible is vital as the cost of this ingredient is the main limiting factor for its use in fish fed thus the species which can use it more efficiently would be the first ones in which this ingredient can be incorporated in their diets (Pratiwy and Pratiwi 2020).

In both species the total fat content of the fish fillets as well as the condition factor of the fish was not significantly changed by the *Schizochytrium* oil levels in the diet the increase growth did not led to excessive fish fattening.

Female gonad maturation

Assessment of the gonad maturation through the Gonado-somatic index (GSI) and vitellogenesis via the Hepato-somatic index (HSI) revealed no alteration caused by the presence of the *Schizochytrium* oil in the diet, with other factors such as the size and sex of the fish significantly affecting those indexes. As expected, female fish had an higher GSI and HIS indexes as this is the norm in mature teleost fish (Bass and Grober 2009). Presence of mature oocytes across all the treatment groups indicated that female fish were able to reach sexual maturity regardless of the diet. There are no previous studies on the gonad development of the Manyara tilapia, but we can draw comparisons with previous work done on Nile tilapia as both species belong to the same genus *Oreochromis*. The effects of the use of ingredients from plant origin on the gonad development in Nile tilapia vary depending which ingredient was used. Kareem, Abdelhadi et al. (2016) found that extracts of *C. papaya* and *A. indica* reduced the GSI and thus delayed gonad maturation of the fish. A decrease of the GSI was also observed when supplementing the tilapia's diet with *A. mossambicensis* and *A. indica* due to the plant anti-nutritional factors (ANF) present in it (Kapinga, Limbu et al. 2019). On the other hand, Ochang, Fagbenro et al. (2007) found no negative effect on the GSI when fish oil was replaced with palm oil. These contradictory results highlight the need for testing the effects of novel ingredients on the gonad development, especially for species due to be breed in captivity. The absence of negative effects on GSI indicated that the correct sexual development of the Manyara tilapia was not altered by the *Schizochytrium* in the diet. The histological analysis which did not detected any effect of the diet on the oocyte's maturation stages either. Future research should investigate the effect of *Schizochytrium* oil on the omega-3 content of Manyara tilapia eggs and the survivability of the fry from broodstock fed using this oil as ingredient. While previous work carried out on Zebrafish (*Danio rerio*) has not provided any evidence of changes to the fatty acid profile of the eggs (Byreddy, Yoganantharjah et al. 2019), no data is currently available on the survivability of fish larvae from parents fed with *Schizochytrium* oil in the diet.

5.6 Conclusions

A healthy and reproductive captive population is a key element to establish successful conservation programmes which include captive breeding for restoking and potentially commercial aquaculture operations, especially when a species never previously farmed like Manyara tilapia is involved. The results of this study showed that this species can reach sexual maturity in captivity with a diet similar to a commercial Nile tilapia diet, and also when conventional fish and plant oils were replaced by novel microalgae oil *Schizochytrium*. Manyara tilapia fed *Schizochytrium* oil grew larger and had a higher omega-3 content in the flesh than control fish, without increasing its total fat content or affecting the normal gonad development. Similarities with the results previously obtained with Nile tilapia indicate that the benefits of *Schizochytrium* oil may be conserved across other members of the *Oreochromis* genus.

CHAPTER 6 – GENERAL DISCUSSION AND CONCLUSIONS

This thesis assessed the effects of *Schizochytrium* oil replacement on the of performance of Nile tilapia (*Oreochromis niloticus*) and Manyara tilapia.

6.1 Quantitative assessment of the benefits from incorporation of Schizochytrium in fish diets

While *Schizochytrium* is a promising candidate to replace fish oil in aquafeeds used in fish farming (Sarker, Kapuscinski et al. 2016, Shah, Lutzu et al. 2018) there is still uncertainty regarding the optimal level of replacement, potential negative effects and the extent to which the benefits of using this microalgae can be generalised across species. What I found out was that replacing fish oil with *Schizochytrium* oil globally did not result in loss of omega-3 content of the fish fillet in most of the trials involving almost all the species examined. This adaptability to fulfil the fatty-acid requirements of most fish species, coupled with the fact that industrial production of *Schizochytrium* oil is already a reality (Tocher, Betancor et al. 2019) suggests this microalgae oil will be the first one to become an ingredient of commercial fish feeds in the near future. The use microalgae in aquafeeds is still more expensive than using fishmeal, fish oils or plant crops, but improvements in the state of the art technologies a could lead to a cost price reduction of 92% in the near future (Oostlander, van Houcke et al. 2020) this in turn will make microalgae oils price competitive, especially considering that the price of marine ingredients has been steadily increasing in the last two decades (Indexmundi 2021). But my thesis also showed that production cost is not the only-gap to address before microalgae in fish feed can become commonplace, rigorous comparative analyses of novel microalgal diets are still needed. Heterogeneity between studies was found to be very high mainly due to differences in the way feeding trials were conducted, those differences may hinder the progress towards a much needed research strategy aligned with industry needs (Turchini, Trushenski et al. 2019). To address the high heterogeneity across the results, feeding trials with *Schizochytrium* should be conducted under commercially relevant conditions. Research papers should also avoid omitting critical experimental details like units of replication, mean effects, sample sizes and measures of variability in other to increase the replicability of the findings.

6.2 Effects of different oil sources on the gut microbiome development of Nile tilapia fry

The early life stage is a key moment in fishes' life during which the gut microbiome develops and its shape by several factors, but prominently by the diet (Nayak 2010). The gut of the fish starts to get colonized by microorganisms primarily at first feeding, however most current studies on the gut microbiota of fish were performed on juvenile fish who were already fed a control diet prior the experiment potentially affecting the results of such studies (Ingerslev, von Gersdorff Jørgensen et al. 2014). In this thesis, I assessed the development of the gut microbiota of Nile tilapia fry from first feeding using 16S sequencing to assess the effects of various levels of microalgae oil replacement. I found that a diet rich in linoleic acid (C18:2 (n-6)) from plant oil promotes the abundance of pathogenic Aeromonadaceae in the gut of Nile tilapia, a bacterial family often associated with intestinal inflammation in the fish gut (Kuebutornye, Wang et al. 2020). Negative effects on the gut microbiota caused by plant oils has already been observed in rainbow trout (Desai, Links et al. 2012), sablefish (Rhodes, Johnson et al. 2016) and it is speculated that this is due to the fact that all natural plant oils are deficient in marine polyunsaturated fatty acids (Merrifield, Olsen et al. 2011). This is also the reason why, when omega-3 rich Schizochytrium oil was used as sole source of fatty acids for Nile tilapia, there was no promotion of Aeromonadaceae. The diets rich in DHA (from fish and microalgae oils) promoted the proliferation of Peptostreptococcaceae like previously observed in Atlantic salmon (Hartviksen, Vecino et al. 2014, Egerton, Wan et al. 2020). I advise toward further research on the potential relation between Peptostreptococcaceae abundance and omega-3 content, as well as studies that clarify the role of this family in the Nile tilapia gut microbiota and how it may affect fish gut health. In addition to not promoting the pathogenic Aeromonadaceae family, diets rich in microalgae oil promoted fish growth and accumulation of omega-3 in the fillet making tilapia more nutritious. The data obtained from this chapter indicates that microalgae oil can be a superior replacement to plant oils already from first feeding making it a sustainable, healthy, and nutritious ingredient for diets targeting juvenile Nile tilapia.

6.3 Long term effects of microalgae oil replacement on Nile tilapia

To characterize, for the first time, the long-term effects of microalgae oil on Nile tilapia (*Oreochromis niloticus*) I raised fish from the previous microbiome experiment on their same

previous diets (control diet with 50:50 fish and plant oil and two diets with increasingly higher microalgae oil replacement levels of 66% and 100% respectively) for eleven months. My results indicate that prolonged use of microalgae Schizochytrium oil as replacement of plant and marine oil can improve growth and enhance omega-3 content to levels comparable to those found among marine fish (Pike and Jackson 2010). While it is well recorded that the inclusion of Schizochytrium in the diet promotes the increase of omega-3 in the fillet of Nile tilapia (Sarker, Gamble et al. 2016, dos Santos, Schorer et al. 2019), the amount of such increase in my thesis was higher than what has been previously recorded. Those results indicate that microalgae-oil enhanced tilapia fillet could represent a sustainable and nutritious alternative to marine fish, especially in countries where tilapia farming is well established and marine fish are not readily available. In addition to improve the nutritional value of the fish, complete Schizochytrium oil replacement did not result in oxidative stress nor did increase mortality, suggesting it has no long-term harmful effects on Nile tilapia.

6.4 Benefits of Schizochytrium oil on Manyara tilapia (*Oreochromis amphimelas*) and comparison with Nile tilapia

Optimal nutrition is a key element to establish successful conservation programmes which include captive breeding but this can be difficult to achieve as nutritional requirements of endangered species are often unknown (Hill, Twibell et al. 2013). In this thesis I grew the endangered Manyara tilapia (*Oreochromis amphimelas*), which is both heavily fished and present only in three lakes of Tanzania (Bradbeer, Harrington et al. 2019), using the same Schizochytrium enriched diets previously used on Nile tilapia in order to evaluate if the benefits recorded in Nile tilapia (better growth and omega-3 content of the fillet) could potentially be transferred to this species. Similarities with the results previously obtained with Nile tilapia were found in Manyara tilapia, indicating that those species share similar nutritional requirements and proves that the benefits of Schizochytrium oil can be transferred to other members of the Oreochromis genus. Manyara tilapia fed on this microalgae oil had an omega-3 content of the fillet comparable with marine fish species (Pike and Jackson 2010, Deepika, Vegneshwaran et al. 2014), demonstrating that *Schizochytrium* oil can be used to enhance the rearing of the endangered Manyara tilapia for conservation and also as a local and sustainable source of omega-3 fatty acids in Tanzania.

APPENDICES

Appendix 1- Supplementary figures and tables

Table S. 1- Proximate analysis (Macronutrients) of the diets of the Nile tilapia (*Oreochromis niloticus*) from Chapter 3. TFA= Total Fatty Acids

Diet	Crude Fibre %	Dry Matter %	Ash %	Total Oil %	Crude Protein %	Carbohydrates %	FFA of extracted fat %	Monounsaturated Fatty Acids % (Of TFA)	Polyunsaturated Fatty Acids % (Of TFA)	Saturated Fatty Acids % (Of TFA)	Unidentified Fatty Acids % (Of TFA)
CON	1.90	92.00	5.00	17.19	33.60	44.21	3.80	26.14	55.97	15.80	2.09
FISH	2.30	85.00	4.60	15.62	30.90	48.88	4.20	30.52	50.64	15.81	3.03
PLANT	2.10	91.10	5.30	16.79	32.70	45.21	4.60	21.90	60.53	16.08	1.49
ALGAE33	2.40	87.30	4.70	16.04	31.90	47.36	3.90	24.57	56.37	16.39	2.67
ALGAE66	3.00	89.20	4.70	15.76	32.50	47.04	4.90	20.97	57.42	17.71	3.90
ALGAE100	2.70	83.70	4.70	15.22	30.50	49.58	28.60	18.80	57.66	18.50	5.04

Table S. 2- Growth performances of the Nile tilapia (*Oreochromis niloticus*) from Chapter 3.

Diet	Tank	Length94 (mm)	Weight21 (gr)	Weight43 (gr)	Weight63 (gr)	Weight94 (gr)	SGR94	Mortality %	K	D94morts	D94alive	Survivability %
CON	1	46.8	0.162	0.364	0.807	1.694	4.53	4	1.66	3	72	96
CON	6	40	0.112	0.386	0.94	1.245	4.2	45.33333	1.95	34	41	54.66667
CON	8	48.8	0.124	0.478	0.746	2.015	4.71	6.666667	1.74	5	70	93.33333
FISH	5	53.5	0.24	0.724	1.042	2.35	4.88	4	1.53	3	72	96
FISH	9	56.5	0.155	0.552	1	2.923	5.11	6.666667	1.62	5	70	93.33333
FISH	15	56.5	0.142	0.886	1.559	2.976	5.13	17.33333	1.65	13	62	82.66667
PLANT	12	40.3	0.15	0.315	0.572	1.148	4.11	17.33333	1.76	13	62	82.66667
PLANT	17	46.3	0.112	0.384	0.693	1.772	4.58	14.66667	1.79	11	64	85.33333
PLANT	18	48	0.122	0.28	0.408	1.792	4.59	9.333333	1.62	7	68	90.66667
ALGAE33	4	52.3	0.163	0.496	0.949	2.906	5.1	8	2.04	6	69	92
ALGAE33	7	53	0.157	0.488	1.014	2.493	4.94	1.333333	1.67	1	74	98.66667
ALGAE33	16	54.8	0.164	0.619	0.968	2.968	5.13	30.66667	1.81	23	52	69.33333
ALGAE66	3	47.5	0.13	0.569	0.945	1.823	4.61	4	1.7	3	72	96
ALGAE66	10	48.3	0.208	0.32	1.208	1.802	4.59	5.333333	1.6	4	71	94.66667
ALGAE66	13	50	0.184	0.757	1.217	2.241	4.83	2.666667	1.79	2	73	97.33333
ALGAE 100	2	48.3	0.167	0.564	1.552	1.96	4.68	28	1.74	21	54	72
ALGAE 100	11	63.5	0.226	1.139	1.836	4.034	5.45	40	1.58	30	45	60
ALGAE 100	14	62.8	0.317	0.803	2.45	4.086	5.47	25.33333	1.65	19	56	74.66667

Table S. 3- Diversity changes in the gut microbiota of the Nile tilapia (*Oreochromis niloticus*) from Chapter 3.

Measure of Diversity	Diet	Time	Mean	L95CI	U95CI
Shannon	Control	21	3.574804	2.9112	4.2384
Shannon	Fish	21	3.320117	2.47736	4.1629
Shannon	Plant	21	4.028738	3.36424	4.6932
Shannon	A33	21	3.453072	2.8186	4.08755
Shannon	A66	21	3.625281	2.94935	4.3012
Shannon	A100	21	3.919388	3.2106	4.6282
Shannon	Control	94	1.693597	1.41884	1.96835
Shannon	Fish	94	1.793307	1.4707	2.1159
Shannon	Plant	94	1.975809	1.61062	2.341
Shannon	A33	94	2.088313	1.8418	2.33483
Shannon	A66	94	1.798273	1.6722	1.92436
Shannon	A100	94	2.336546	2.0647	2.6084
Chao	Control	21	98.44574	71.736	125.156
Chao	Fish	21	83.17726	47.455	118.9
Chao	Plant	21	104.091	72.658	135.524
Chao	A33	21	75.99727	54.174	97.82
Chao	A66	21	84.90433	55.67	114.139
Chao	A100	21	101.429	63.319	139.54
Chao	Control	94	68.2222	28.774	107.67
Chao	Fish	94	53.742	32.128	75.356
Chao	Plant	94	70.82663	49.253	92.4
Chao	A33	94	83.97276	54.398	113.547
Chao	A66	94	54.95952	37.562	72.357
Chao	A100	94	79.45584	52.967	105.945

Table S. 4- Growth performances of the Nile tilapia (*Oreochromis niloticus*) from Chapter 4. SGR= Standard Growth Rate, CF= Condition Factor

Sample Number	FISH-ID	Tank	DIET	Weight (gr)	Fork Length (cm)	SGR	CF
1	CON-1	1	CON	343.6	27.3	1.085414	1.688749
2	CON-2	1	CON	170.2	21	1.083401	1.837814
3	CON-3	1	CON	327.1	26.3	1.085273	1.798098
4	CON-4	1	CON	337.5	25.9	1.085363	1.94256
5	CON-5	1	CON	223.2	23.5	1.084178	1.71985
6	CON-6	1	CON	286.8	25.5	1.084897	1.729651
7	A50-1	2	A50	320	24.2	1.085211	2.257896
8	A50-2	2	A50	335	26.8	1.085342	1.740365
9	A50-3	2	A50	370.7	26.9	1.085632	1.904433
10	A50-4	2	A50	337	26	1.085359	1.917387
11	A50-5	2	A50	303.6	25.7	1.08506	1.788555
12	A50-6	2	A50	272.4	24.5	1.084749	1.852289
13	A100-1	3	A100	453	29.4	1.086206	1.782609
14	A100-2	3	A100	249.3	24	1.084495	1.803385
15	A100-3	3	A100	457.6	28.5	1.086235	1.976749
16	A100-4	3	A100	460.7	28	1.086255	2.09867
17	A100-5	3	A100	278.2	23.5	1.084809	2.143648
18	A100-6	3	A100	436.4	28.3	1.086099	1.92542
19	CON-7	4	CON	266.1	23.5	1.084682	2.050413
20	CON-8	4	CON	277.3	24	1.0848	2.005932
21	CON-9	4	CON	291	24.8	1.084938	1.907822
22	CON-10	4	CON	319.6	25.5	1.085207	1.927464
23	CON-11	4	CON	280.6	24.1	1.084834	2.004641
24	CON-12	4	CON	431.9	28	1.08607	1.967474
25	A100-7	5	A100	540.8	28.5	1.086714	2.336157
26	A100-8	5	A100	383.3	27	1.085728	1.947366
27	A100-9	5	A100	235.5	23	1.084332	1.935563
28	A100-10	5	A100	507.2	29.5	1.08653	1.975665
29	A100-11	5	A100	373.5	26.9	1.085653	1.918818
30	A100-12	5	A100	407.3	28	1.085902	1.855412
31	A50-7	6	A50	277.5	26.6	1.084802	1.474409
32	A50-8	6	A50	374	25.9	1.085657	2.152644
33	A50-9	6	A50	276.7	23.8	1.084794	2.052477
34	A50-10	6	A50	332.3	26	1.085319	1.890646
35	A50-11	6	A50	265.4	23.4	1.084674	2.071349
36	A50-12	6	A50	339.6	26.5	1.085381	1.824862

Table S. 5- Omega-3, fat content and gene expression of the Nile tilapia (*Oreochromis niloticus*) from Chapter 4.

FISH ID	Tank	Diet	tot Omega3 %	total fat %	cat	elovl5	lpl
CON-3	1	CON	21.55	3.9	0.021419	0.001439	0
CON-4	1	CON	20.89	5	0.026561	0.001458	0
CON-5	1	CON	21.24	3	0.074399	0.001441	0.002168
A50-1	2	A50	27.1	3.4	0.049554	0.011721	0.003345
A50-2	2	A50	27.21	6	0.076591	0.000509	0
A50-3	2	A50	24.2	4.8	0.050332	0.003752	0.00062
A100-1	3	A100	31.95	6.4	0.046953	0.011293	0.000432
A100-3	3	A100	32.77	6.1	0.023701	0.018619	0.004181
A100-4	3	A100	33.64	6.9	0.02775	0.003257	0
CON-7	4	CON	23.13	7.4	0.031367	0.011791	0
CON-8	4	CON	22.53	5.2	0.016737	0.001911	0
CON-9	4	CON	22.48	5.2	0.030923	0.000842	0.000153
A100-7	5	A100	30.75	7.8	0.0221	0.001773	0.001019
A100-10	5	A100	31.46	6.9	0.023587	0.003605	0.003103
A100-11	5	A100	33.37	5.2	0.022519	0.004377	0.000735
A50-7	6	A50	27.33	4.9	0.020431	0.000286	0
A50-10	6	A50	25.62	6.3	0.018443	0.006648	0
A50-11	6	A50	27.53	3.9	0.03989	0.005387	0

Table S. 6- Growth performances of the Manyara tilapia (*Oreochromis amphimelas*) from Chapter 5. GSI= Gonado-Somatic Index, HIS= Hepato-Somatic Index, SGR= Standard Growth Rate, CF= Condition factor.

Fish ID	Tank	Diet	Total weight (gr)	Gonad weight (gr)	Liver weight (gr)	Sex	total length (mm)	GSI	HSI	SGR	CF
1	1	CON	1.91	0.026	0.082	F	53	1.361257	4.293194	3.622649	1.282938
2	1	CON	2.37	0.025	0.038	M	54	1.054852	1.603376	3.625218	1.505106
3	1	CON	2.5	0.027	0.067	M	56	1.08	2.68	3.625854	1.42356
4	1	CON	2.98	0.057	0.038	M	60	1.912752	1.275168	3.627945	1.37963
5	1	CON	1.74	0.03	0.038	F	49	1.724138	2.183908	3.621539	1.478976
6	1	CON	2.05	0.019	0.045	M	52	0.926829	2.195122	3.623491	1.457954
7	1	CON	2.44	0.038	0.071	M	54	1.557377	2.909836	3.625565	1.549561
8	1	CON	1.49	0.06	0.053	F	48	4.026846	3.557047	3.619693	1.347295
9	1	CON	2.11	0.032	0.074	M	53	1.516588	3.507109	3.623835	1.417277
10	1	CON	1.81	0.138	0.068	F	49	7.624309	3.756906	3.622009	1.538475
11	2	CON	1.96	0.013	0.063	M	52	0.663265	3.214286	3.622957	1.393946
12	2	CON	2.08	0.011	0.035	M	52	0.528846	1.682692	3.623664	1.47929
13	2	CON	2.43	0.025	0.058	M	55	1.028807	2.386831	3.625516	1.460556
14	2	CON	1.64	0.008	0.018	M	47	0.487805	1.097561	3.620835	1.579611
15	2	CON	1.59	0.008	0.049	M	48	0.503145	3.081761	3.620466	1.437717
16	2	CON	2.29	0.03	0.032	M	54	1.310044	1.39738	3.624809	1.454301
17	2	CON	2.89	0.05	0.072	M	58	1.730104	2.491349	3.62758	1.481201
18	2	CON	2.4	0.078	0.087	F	53	3.25	3.625	3.625368	1.612069
19	2	CON	1.57	0.002	0.047	M	49	0.127389	2.993631	3.620316	1.334478
20	2	CON	2.09	0.017	0.054	M	55	0.813397	2.583732	3.623721	1.256198
21	3	A100	2.82	0.053	0.109	F	57	1.879433	3.865248	3.627288	1.522736
22	3	A100	3.29	0.058	0.077	M	62	1.762918	2.340426	3.629123	1.38045
23	3	A100	1.93	0.037	0.051	M	51	1.917098	2.642487	3.622773	1.454946
24	3	A100	2.22	0.103	0.053	F	54	4.63964	2.387387	3.62444	1.409846
25	3	A100	2.53	0.092	0.056	F	55	3.636364	2.213439	3.625996	1.520661

26	3	A100	3.21	0.225	0.099	F	58	7.009346	3.084112	3.62883	1.645209
27	3	A100	1.65	0.031	0.031	M	46	1.878788	1.878788	3.620907	1.695159
28	3	A100	1.59	0.008	0.049	F	48	0.503145	3.081761	3.620466	1.437717
29	3	A100	2.44	0.053	0.053	M	55	2.172131	2.172131	3.625565	1.466566
30	3	A100	3.44	0.051	0.108	M	62	1.482558	3.139535	3.629654	1.443389
31	4	A50	2.2	0.054	0.041	M	53	2.454545	1.863636	3.624332	1.47773
32	4	A50	2.12	0.026	0.027	F	54	1.226415	1.273585	3.623891	1.346339
33	4	A50	2.29	0.024	0.072	F	53	1.048035	3.144105	3.624809	1.538183
34	4	A50	2.97	0.014	0.067	F	60	0.47138	2.255892	3.627905	1.375
35	4	A50	2.67	0.04	0.085	M	56	1.498127	3.183521	3.626637	1.520363
36	4	A50	1.45	0.003	0.049	F	46	0.206897	3.37931	3.619369	1.489685
37	4	A50	2.5	0.067	0.044	M	56	2.68	1.76	3.625854	1.42356
38	4	A50	2.3	0.043	0.061	M	53	1.869565	2.652174	3.624861	1.544899
39	4	A50	1.59	0.008	0.04	M	46	0.503145	2.515723	3.620466	1.633517
40	4	A50	3.36	0.086	0.082	M	60	2.559524	2.440476	3.629373	1.555556
41	5	CON	2.56	0.184	0.056	F	54	7.1875	2.1875	3.626136	1.625768
42	5	CON	2.34	0.043	0.054	M	54	1.837607	2.307692	3.625066	1.486054
43	5	CON	2.76	0.046	0.035	M	56	1.666667	1.268116	3.627032	1.571611
44	5	CON	2.3	0.058	0.071	F	51	2.521739	3.086957	3.624861	1.733873
45	5	CON	2.54	0.032	0.047	M	55	1.259843	1.850394	3.626043	1.526672
46	5	CON	2	0.128	0.069	F	51	6.4	3.45	3.623197	1.507716
47	5	CON	2.09	0.038	0.065	F	53	1.818182	3.110048	3.623721	1.403843
48	5	CON	1.58	0.023	0.035	F	49	1.455696	2.21519	3.620391	1.342978
49	5	CON	2.82	0.066	0.084	M	58	2.340426	2.978723	3.627288	1.445324
50	5	CON	1.41	0.011	0.036	F	47	0.780142	2.553191	3.619036	1.358081
51	6	A50	2.05	0.01	0.069	F	52	0.487805	3.365854	3.623491	1.457954
52	6	A50	3.1	0.153	0.116	F	57	4.935484	3.741935	3.628415	1.673929
53	6	A50	1.55	0.016	0.0048	F	47	1.032258	0.309677	3.620163	1.492925
54	6	A50	3.32	0.049	0.073	F	60	1.475904	2.198795	3.629231	1.537037
55	6	A50	2.52	0.034	0.0045	M	57	1.349206	0.178571	3.625949	1.360743
56	6	A50	1.22	0.007	0.046	F	43	0.57377	3.770492	3.617313	1.534456

57	6	A50	1.16	0.007	0.026	F	41	0.603448	2.241379	3.616712	1.683086
58	6	A50	2.87	0.029	0.089	F	57	1.010453	3.101045	3.627497	1.549735
59	6	A50	2.44	0.064	0.052	F	55	2.622951	2.131148	3.625565	1.466566
60	6	A50	2.26	0.045	0.04	M	53	1.99115	1.769912	3.624652	1.518032
61	7	A50	2.33	0.239	0.084	F	52	10.25751	3.60515	3.625015	1.657089
62	7	A50	2.89	0.092	0.057	M	60	3.183391	1.972318	3.62758	1.337963
63	7	A50	2.23	0.045	0.07	F	54	2.017937	3.139013	3.624493	1.416197
64	7	A50	1.97	0.028	0.053	F	49	1.42132	2.690355	3.623017	1.674472
65	7	A50	2.6	0.06	0.059	M	55	2.307692	2.269231	3.626321	1.562735
66	7	A50	1.52	0.03	0.031	M	46	1.973684	2.039474	3.61993	1.561601
67	7	A50	2.62	0.062	0.048	M	58	2.366412	1.832061	3.626412	1.342818
68	7	A50	1.53	0.015	0.042	M	48	0.980392	2.745098	3.620008	1.383464
69	7	A50	1.87	0.147	0.031	F	50	7.860963	1.657754	3.622397	1.496
70	7	A50	2.27	0.051	0.0053	M	52	2.246696	0.23348	3.624705	1.614417
71	8	A100	3.21	0.058	0.09	M	60	1.806854	2.803738	3.62883	1.486111
72	8	A100	3.03	0.03	0.085	M	60	0.990099	2.805281	3.628143	1.402778
73	8	A100	3.39	0.061	0.099	M	62	1.79941	2.920354	3.629479	1.422409
74	8	A100	2.85	0.032	0.096	M	57	1.122807	3.368421	3.627414	1.538935
75	8	A100	1.99	0.018	0.058	F	51	0.904523	2.914573	3.623138	1.500177
76	8	A100	3.01	0.122	0.086	F	59	4.053156	2.857143	3.628064	1.465583
77	8	A100	1.42	0.048	0.045	F	48	3.380282	3.169014	3.61912	1.283999
78	8	A100	1.32	0.016	0.036	M	45	1.212121	2.727273	3.618251	1.44856
79	8	A100	2.12	0.134	0.067	F	53	6.320755	3.160377	3.623891	1.423994
80	8	A100	2.25	0.033	0.071	M	51	1.466667	3.155556	3.624599	1.69618
81	9	A100	2.07	0.019	0.058	F	52	0.917874	2.801932	3.623607	1.472178
82	9	A100	5.33	0.129	0.136	M	71	2.420263	2.551595	3.634866	1.489197
83	9	A100	1.19	0.018	0.042	M	44	1.512605	3.529412	3.617016	1.396976
84	9	A100	5.86	0.134	0.161	M	73	2.286689	2.74744	3.635995	1.506361
85	9	A100	4.8	0.055	0.095	M	70	1.145833	1.979167	3.63362	1.399417
86	9	A100	4.12	0.107	0.061	M	67	2.597087	1.480583	3.631801	1.369849
87	9	A100	2.12	0.03	0.042	F	53	1.415094	1.981132	3.623891	1.423994

88	9	A100	2.77	0.083	0.099	F	60	2.99639	3.574007	3.627075	1.282407
89	9	A100	1.26	0.006	0.041	M	42	0.47619	3.253968	3.617697	1.70068
90	9	A100	4.31	0.083	0.103	M	67	1.925754	2.389791	3.632338	1.433022

Table S. 7- Omega-3 and fat content of the fillet of the Manyara tilapia (*Oreochromis amphimelas*) from Chapter 5.

Tank	Diet	Omega3 (%)	Fat (%)
1	CON	19.02	9.6
2	CON	19.51	10
3	A100	32.25	10.9
4	A50	26.9	10.9
5	CON	18.39	10.3
6	A50	26.36	10.5
7	A50	27.5	10
8	A100	32.68	9.7
9	A100	32.71	10.8

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